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**ANALYSIS OF THE VIRULENCE REGULATORY  
LOCUS OF *BORDETELLA PERTUSSIS*.**

Thesis presented for the degree of  
Doctor of Philosophy,  
Faculty of Science,  
Department of Microbiology,  
University of Glasgow,  
September 1990.

Doreen M. McGillivray, B.Sc. (Hons.)



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and yet-  
and yet-

*Issa*

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## PUBLICATIONS.

### Abstracts.

McGillivray, D. M., Brownlie, R. M., Parton, R. and Coote, J. G. Complementation of avirulent strains of *Bordetella pertussis* by *B.pertussis* DNA cloned in broad-host-range vectors. FEMS-Symposium PERTUSSIS, Berlin, April 20- 22, 1988.

McGillivray, D. M., Coote, J. G. and Parton, R. Studies on the virulence regulatory *vir* gene of *Bordetella pertussis*. International Workshop on *Bordetella pertussis*, Hamilton, Montana, August 18- 20, 1988.

McGillivray, D. M., Coote, J. G. and Parton, R. The virulence regulatory *vir* gene of *Bordetella pertussis*. Society for General Microbiology, 113th Ordinary Meeting, Cambridge, April 4- 7, 1989.

### Papers.

McGillivray, D. M., Coote, J. G. and Parton, R. (1989). Cloning of the virulence regulatory (*vir*) locus of *Bordetella pertussis* and its expression in *B.bronchiseptica*. FEMS Microbiology Letters. 65, 333- 338.

# ABBREVIATIONS.

Ab	antibody
AC	adenylate cyclase
Adc	phenotype for AC
ADP	adenosine diphosphate
AGG	agglutinin
Ap	ampicillin
ATP	adenosine triphosphate
BG	Bordet-Gengou
bp	base pairs
BSA	bovine serum albumin
<i>bvg</i>	<i>Bordetella</i> virulence gene, also known as <i>vir</i>
CaM	calmodulin
cAMP	cyclic 3', 5' adenosine monophosphate
CAMR	Centre for Applied Microbiology and Research
CAT	Chloramphenicol acetyltransferase
<i>cat</i>	genotype for CAT
CIP	calf intestinal alkaline phosphatase
contd.	continued
<i>cya</i>	genotype for AC
CR	Congo red
d.H <sub>2</sub> O	distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FHA	filamentous haemagglutinin
Fha	phenotype for FHA
<i>fha</i>	genotype for FHA
<i>fim</i>	genotype for fimbriae
g	grammes
GTP	Guanosine triphosphate
h	hour
HLT	heat-labile toxin
HLY	haemolysin
Hly	phenotype for HLY
<i>hly</i>	genotype for HLY
ic	intracerebral
ICMPT	intracerebral challenge mouse protection test
iFHA	immunologically reactive FHA
IPTG	isopropyl- $\beta$ - D- thiogalactopyranoside
IS	insertion sequence
kb	kilobase pairs
kD	kilo Daltons
Km	kanamycin
l	litre
LPS	lipopolysaccharide
M	Molar
mA	milli Amperes
MAB	monoclonal antibody
MeCD	Hepatkis 2,6- O- dimethyl $\beta$ -cyclodextrin

mg	milligrammes
min	minute
ml	millilitres
mM	milli Molar
<i>mod</i>	modulation gene
MRC	Medical Research Council
mRNA	messenger RNA
NAD	nicotinamide-adenine dinucleotide
nm	nanometres
nM	nanomoles
OD	optical density (absorbance)
ORF	open reading frame
OU	opacity units
PBS	phosphate buffered saline
PEG	polyethylene glycol
PMSF	phenylmethanesulphonyl fluoride
PT	pertussis toxin
Ptx	phenotype for PT
<i>ptx</i>	genotype for PT
RNA	ribonucleic acid
r.p.m.	revolutions per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	second
SS	Stainer and Scholte
ST	serotype
TAE	Tris-acetate buffer
TBE	Tris-borate buffer
Tc	tetracycline
TCT	tracheal cytotoxin
TE	Tris-EDTA buffer
temp	temperature
Tn	transposon
u.v.	ultra violet
V	Volts
<i>vag</i>	<i>vir</i> -activated gene
Vir	phenotype for virulence-regulatory protein
<i>vir</i>	genotype for virulence-regulatory protein, also known as <i>bvg</i>
<i>vrg</i>	<i>vir</i> -repressed gene
WHO	World Health Organization
X-gal	5- bromo- 4- chloro- 3- indolyl- $\beta$ - D- galactopyranoside
X-OMPs	X-mode specific envelope polypeptides
pmol	picomoles
$\mu$ g	microgrammes
$\mu$ l	microlitres
% w/v	weight in grammes per 100ml water
% v/v	volume in ml per 100ml water
::	novel joint

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Section 1: SUMMARY.

The primary aim of this research was to characterize recombinant cosmid clone pRMB2, and subclone the *vir* locus. Clone pRMB2 was identified from a gene library of *Bordetella pertussis* TAB I DNA in *Escherichia coli*, by its ability to restore a Vir+ phenotype to Vir- Tn5 mutant *B.pertussis* BP347 (Brownlie et al., 1988).

Restriction analysis with *EcoRI* revealed that the genomic insert of pRMB2 contained six fragments, designated E1 (1.1kb), E2 (2.5kb), E3 (2.7kb), E4 (4.7kb), E5 (5.1kb) and E6 (10.0kb). Hybridisation with pRMB2 and E3 probes showed that the Tn5 insertion in Vir- BP347 was located within an *EcoRI* fragment of 2.7kb. The inactivation of this region by Tn5 indicated that it was essential to the *vir* region.

The E3 fragment of pRMB2 was subcloned in pLAFR1 to give a construct designated pDM3. In-trans, pDM3 was unable to complement Vir- BP347, which implied that the 2.7kb *EcoRI* fragment contained insufficient sequence to encode an active *vir* locus. In a small percentage of BP347 (pDM3) transconjugants, homologous recombination had occurred in the *vir* region, such that a fully functional *vir* locus was restored to BP347 with an attendant gain of expression of virulence-associated determinants.

Clone pRMB2 was partially digested with *Sau3A* to generate random fragments of approximately 6kb. Ligation of these fragments into vector pRK310 yielded forty-eight recombinant clones, none of which contained sufficient sequence to complement Vir- BP347.

Hybridisation analysis of a series of restriction digests of pRMB2 with E3 identified three putative *vir*-containing fragments, which each consisted of pLAFR1 vector and the terminal portions of the genomic insert of pRMB2. One of these fragments was ligated to give a construct designated pDM1 which, in-trans, restored a Vir+ phenotype to Vir- BP347. Restriction

enzyme analysis of pDM1 showed that the genomic insert comprised two non-contiguous fragments of 3.9kb and 8.0kb. The E3 fragment was contained within the latter, which demonstrated that this 8.0kb region encoded a functional *vir* locus.

A restriction map was compiled for clone pRMB2, and comparison with a published map for the *vir* and *fha* region (Stibitz *et al.*, 1988a) showed homology for a 15.2kb region (E3, E2 and E6) encoding *vir* and *fhaB*, but the remaining 10.9kb (E5, E4 and E1) of pRMB2 showed a different restriction pattern. Hybridisation analysis confirmed that the genomic insert of pRMB2 comprised two non-contiguous regions of TAB I genomic DNA. Clone pRMB2 was unable to complement BP353 (Fha-) which contained a Tn5 insertion in *fhaA*, just downstream of *fhaB*.

Hybridisation with pRMB2 and E3 probes was used to analyse regions homologous to *vir* in a number of strains of all four *Bordetella* species. These studies indicated that the *vir* locus was conserved in *B.pertussis*, *B.parapertussis* and *B.bronchiseptica*. However, the structure of the *vir* region in the latter two species differed from that of *B.pertussis*. Avirulent phase variant *B.pertussis* 11615 had a rearrangement in the *vir* region. *B.avium* chromosomal DNA also showed homology to the *B.pertussis* *vir* locus, but a markedly different hybridisation pattern was observed. This confirmed that *B.avium* was genetically divergent from the other *Bordetella* species. A slight variation in the hybridisation pattern was observed between virulent and avirulent *B.avium* strains.

In-*trans* the *vir* locus encoded by pDM1 was able to restore a Vir+ phenotype to avirulent phase variant strains of *B.pertussis* and *B.bronchiseptica*. This indicated that phase variation in both these species had occurred as a result of genotypic changes in *vir*. However, expression

of virulence determinants in avirulent phase variant strains of *B.bronchiseptica* was restored to only a low level by pDM1, which implied that the mechanisms of regulation of expression of virulence determinants were different for the two species.

The effect of multiple copies of *vir* in-*trans* in *B.pertussis* on the response to modulators was investigated. Multiple copies of *vir*, in clones pRMB2 and pDM1, had no effect on the response to modulation by low temperatures. Tohama-derived strains, including BP347 (pRMB2) and BP347 (pDM1) were resistant to modulation by nicotinic acid.

The ability of *vir* to *trans*-activate expression in *E.coli* of *B.pertussis* virulence genes was investigated: no expression of *fhaB*, *fim2*, *fim3* or *cya* was detected.

Section 2: INTRODUCTION.

## Section 2.1. THE GENUS BORDETELLA.

### 2.1.1. Classification.

*Bordetella pertussis*, *B.parapertussis*, *B.bronchiseptica* and, the newest accession, *B.avium* (Kerstens *et al.*, 1984) currently comprise the genus *Bordetella*. Further information on the genus and its species is given by Olson (1975), Linneman and Perry (1977), Goodnow (1980), Pittman and Wardlaw (1981), Switzer and Farrington (1981), Pittman (1984), Wardlaw (1988) and Wardlaw and Parton (1988a).

The pertussis bacillus was first isolated by Bordet and Gengou (1906). Originally, blood was considered necessary for its cultivation, and it was listed in the genus *Haemophilus* (Winslow *et al.*, 1920; Bergey *et al.*, 1923). However, Fildes (1923) showed that by generic definition, the pertussis bacillus was excluded from the genus *Haemophilus*. Like pertussis, the parapertussis bacillus was initially placed in the genus *Haemophilus* (Bradford and Slavin, 1937; Eldering and Kendrick, 1937, 1938). When first isolated, *B.bronchiseptica* was thought to be the causal agent of canine distemper and it was identified as *Bacillus bronchicanis* (Ferry, 1911, 1912). In the ensuing years, the bronchiseptica bacillus was placed in the genera *Alcaligenes*, *Brucella* and *Haemophilus* (Bergey *et al.*, 1925; Topley and Wilson, 1929; Wilson and Miles, 1946; Haupt, cited by Pittman, 1974).

Classification of these three organisms in a new genus, *Bordetella*, was proposed by Moreno Lopez (1952), and accepted in the 7<sup>th</sup> and 8<sup>th</sup> editions of *Bergey's Manual of Determinative Bacteriology* (Pittman, 1957, 1974). Proom (1955) found that their nutritional requirements were similar, but quite different from organisms of the genera *Haemophilus* or *Brucella*, which supported the view of Moreno Lopez that the three groups should be

classified in a separate genus.

#### 2.1.2. Diseases caused by the bordetellae, and species specificity.

The bordetellae cause respiratory diseases with many similarities in their respective hosts. Bordetellosis is characterized by a primary, localized infection of the ciliated tracheal epithelial cells. After an initial colonization of the ciliated respiratory tract epithelium, there follows a loss of ciliated cells, excessive mucus production, reduced weight gain of the host, and some form of cough or sneezing. Secondary infections are another common feature. The young of the host species are very susceptible to infection.

*B.pertussis* exhibits an exclusive species specificity, infecting only man, and is the aetiological agent of pertussis (whooping cough). *B.parapertussis* is the agent of parapertussis (a milder disease than pertussis) and was, until recently, regarded as a specifically human parasite. However, the isolation of *B.parapertussis* from lambs has been reported (Chen *et al.*, cited by Parton, 1989). *B.bronchiseptica* parasitizes a broad spectrum of both domestic and wild animals e.g. pig, dog, cat, rabbit, rat, horse, monkey, turkey, and occasionally man (see Pittman and Wardlaw, 1981). The diseases caused by *B.bronchiseptica* include atrophic rhinitis in swine (Switzer, 1956) and kennel cough in dogs (Wright *et al.*, 1973; Thompson *et al.*, 1976). *B.avium* is the causal agent of turkey coryza or rhinotracheitis, and has been isolated from turkeys, chickens, ducks and geese (Kersters *et al.*, 1984).

Adherence interaction between bordetellae and ciliated respiratory epithelium is a central process in the pathogenesis of bordetellosis, and may offer an explanation for differences in species specificity of the

bordetellae. *In vitro* studies of the abilities of the bordetellae to adhere to various ciliated epithelial cells appear to parallel the species specificity of the natural infection (Tuomanen, 1988). In a study of the adhesion of the mammalian bordetellae to human cilia, *B.pertussis* adhered best, and *B.bronchiseptica* adhered least well. *B.bronchiseptica* and, surprisingly, *B.parapertussis* adhered best to non-human mammalian cilia (Tuomanen *et al.*, 1983). *B.avium* shows an adherence specificity for turkey cilia (Gray *et al.*, 1983; Arp and Cheville, 1984).

### 2.1.3. Characteristics of the bordetellae.

The bordetellae are a group of Gram-negative, obligate bacterial pathogens, with many features in common (see Table 2.1). More detailed information on certain characteristics of *Bordetella* species is given in Section 2.3. The bordetellae are unable to ferment carbohydrates. The mammalian bordetellae at least, have a requirement for nicotinamide or nicotinic acid, cysteine and methionine (Hornibrook, 1940; Pittman, 1974). *B.avium* strains are reported to have a significantly different fatty acid composition from that of the other *Bordetella* species (Jackwood *et al.*, 1986). *B.pertussis* and *B.parapertussis* are non-motile species, whereas *B.bronchiseptica* and *B.avium* are motile.

Possession of certain virulence-associated determinants is shared by members of the genus *Bordetella* (see Table 2.1). For recent reviews on *B.pertussis* virulence factors, see Wardlaw and Parton (1983a), Weiss and Hewlett (1986), Wardlaw and Parton (1988b), and Parton (1989).

The mammalian bordetellae possess a common heat-stable 'O' antigen, and various heat-labile 'K' antigens (agglutinogens, AGGs) (Andersen, 1953). These surface antigens elicit antibodies which can cause bacterial cell



Table 2.1.

Characteristics of the *Bordetella* species

Characteristic	<i>Bp</i>	<i>Bpp</i>	<i>Bb</i>	<i>Ba</i>
Bordet-Gengou agar growth. No. of days for colony appearance	3 - 6	2 - 3	1 - 2	1 - 2
citrate utilized	-	+	+	n.a.
urease produced	-	+	+	n.a.
peptone agar-browning	-	+	-	n.a.
motility	-	-	+	+
agglutinogens : species specific				
1	+	-	-	n.a.
12	-	-	+	n.a.
14	-	+	-	n.a.
filamentous haemagglutinin	+	+	+	n.a.
pertussis toxin	+	-	-	-
adenylate cyclase	+	+	+	-
haemolysin	+	+	+	-
heat-labile toxin	+	+	+	+
tracheal cytotoxin	+	+	+	+
lipopolysaccharide endotoxin	+	+	+	+
modulation	+	+	+	+/-
phase variation	+	+	+	n.a.
G + C content of DNA (mol %)	67.7-68.0	68.1-69.0	68.2-69.5	61.6-62.6

n.a. = not available

Data obtained from Pittman (1974, 1984); Wardlaw and Parton (1988a).

*Bp*, *B. pertussis*; *Bpp*, *B. parapertussis*; *Bb*, *B. bronchiseptica*; *Ba*, *B. avium*.

agglutination, if the antigens are present in sufficient density for bivalent antibody to cross-link two bacteria. Agglutinogens 1, 14 and 12 are specific for *B.pertussis*, *B.parapertussis* and *B.bronchiseptica* respectively, and AGG 7 is common to at least the three mammalian species (Eldering et al., 1957). Kersters et al. (1984) reported that *B.avium* also possesses the common heat-stable 'O' antigen, and shares heat-labile AGGs with *B.bronchiseptica*. With antisera raised against *B.pertussis* AGG 2 and AGG 3 subunits, Mooi et al. (1987) detected serologically related polypeptides in the other *Bordetella* species, indicating that all four species possess common antigenic determinants on their fimbriae. Filamentous haemagglutinin (FHA), a potential adhesin, is present at least in the mammalian bordetellae.

All species of the genus *Bordetella* produce a similar, but not identical, range of toxins. *B.pertussis* is the only member of the genus to produce pertussis toxin (PT). The metabolic burden of synthesizing this protein could perhaps account for the slower growth rate of *B.pertussis* compared with other members of the genus. Adenylate cyclase (AC) activity is found only in *B.pertussis*, *B.parapertussis* and *B.bronchiseptica*. The three mammalian bordetellae also produce haemolysin (HLY), which is part of the AC toxin complex. Production of heat-labile toxin (HLT), tracheal cytotoxin (TCT) and lipopolysaccharide (LPS; endotoxin) is common to all four *Bordetella* species. This conservation suggests that HLT, TCT and LPS are important virulence factors in bordetellosis (Gentry-Weeks et al., 1988; Cookson et al., 1989).

The phenomena of phenotypic modulation and phase variation are well documented for *B.pertussis*, and are reported in Section 2.4. Phenotypic modulation (Lacey, 1960; Pusztai and Joo, 1967) is due to reversible

phenotypic changes in response to environmental signals such as temperature, magnesium sulphate or nicotinic acid. Phase variation (Leslie and Gardner, 1931) is due to genotypic changes. Both these processes give rise to cells which no longer express virulence determinants. *B.parapertussis* and *B.bronchiseptica* also exhibit phenotypic modulation and phase variation (Ezzell *et al.*, 1981a, b; Peppler and Schrumpf, 1984<sup>a,b</sup> Lax, 1985; Ishikawa and Isayama, 1986; Parton, 1988). It is not clear whether *B.avium* undergoes the phenotypic modulation characteristic of the other bordetellae. Gentry-Weeks *et al.* (1988) reported that production of HLT in *B.avium* was significantly decreased when grown in the presence of a high concentration of MgSO<sub>4</sub>, but only slightly decreased when grown in the presence of a high concentration of nicotinic acid, and not altered by growth at low temperatures. However, Parton (1988) found that expression of HLT by *B.avium* was not affected by growth on high-nicotinic acid medium.

#### 2.1.4. Genetic relatedness and evolution of the bordetellae.

Although the mammalian bordetellae are readily distinguishable by cultural characteristics and pathogenicity (Pittman, 1984), DNA-DNA and DNA-rRNA hybridisation analyses have indicated that the three species show a high degree of DNA homology, perhaps too high to justify their separate classification (Kloos *et al.*, 1979, 1981; Kersters *et al.*, 1984). Musser *et al.* (1986) analysed, by electrophoresis, allelic variation at structural genes encoding fifteen metabolic enzymes for sixty *Bordetella* strains. It was concluded that *B.parapertussis* and *B.bronchiseptica* share a very close genetic relationship, with *B.pertussis* showing only limited divergence. A further study of metabolic enzyme polymorphism in a large number of *B.bronchiseptica* isolates (Musser *et al.*, 1987) showed that strains could

broadly be divided into five groups, each being mainly associated with a different host species. From this study, Musser *et al.* (1987) proposed that *B.bronchiseptica* was probably ancestral to *B.parapertussis* and *B.pertussis*.

Investigations by Arico and Rappuoli (1987), and Marchitto *et al.* (1987a), surprisingly revealed that *B.bronchiseptica* and *B.parapertussis* contain structural genes for PT. However, due to mutations mainly in the promoter region, these genes are not transcriptionally active in either of the two species (Arico and Rappuoli, 1987). Comparisons of the *ptx* gene promoter regions of various *Bordetella* strains with a published sequence for this region in *B.pertussis* strain 165 (Nicosia *et al.*, 1986), showed that *B.parapertussis* and *B.bronchiseptica* have many common point mutations, suggesting that they may derive from a common ancestor (Arico *et al.*, 1987). In agreement with Musser *et al.* (1986), Arico *et al.* (1987) found that strains of *B.pertussis* were genotypically homogeneous and likely to derive from the same clone.

It has been proposed that *B.parapertussis* could represent a non-virulent form of *B.pertussis* (Granstrom and Askelof, 1982; Loch and Keith, 1987) and, under certain conditions *in vitro*, one species converts to the other (Kumazawa and Yoshikawa, 1978; Mebel *et al.*, 1985). However, genetic studies have shown that the proposed conversion appears highly improbable (Musser *et al.*, 1986; Arico *et al.*, 1987).

The standard challenge strain of *B.pertussis*, 18323 (Pittman, 1984), used in the intracerebral mouse protection test (ICMPT, Kendrick *et al.*, 1947) has been reported to be genetically closer to *B.bronchiseptica* and *B.parapertussis*, than to *B.pertussis* (Musser *et al.*, 1986; Arico *et al.*, 1987). Some of the mutations common to *B.bronchiseptica* and *B.parapertussis* in the promoter region and structural gene for the S1 subunit of PT, are

also found in *B.pertussis* 18323 (Arico *et al.* 1987). The S1 subunit produced by strain 18323, and recombinant S1 subunits produced by *B.parapertussis* and *B.bronchiseptica*, are antigenically different from the same subunit produced by other *B.pertussis* strains (Perera *et al.*, 1986; Arico *et al.*, 1987).

*B.pertussis* has been shown to carry a repeated DNA sequence, present in multiple copies in the genome (McPheat and McNally, 1987a). This sequence is specific to *B.pertussis*, and absent from the other *Bordetella* species (McPheat and McNally, 1987b). Sequence analysis of two copies of the repeated unit showed that they were variants of an insertion sequence (IS) element identified by McLafferty *et al.* (1988) (McPheat *et al.*, 1989). A repeated DNA unit specific for both *B.pertussis* and *B.parapertussis* has also been found (McLafferty *et al.*, 1988).

*B.avium* was found to be significantly genetically divergent from the other *Bordetella* species, sufficient to merit recognition as a separate species (Kerstens *et al.*, 1984; Musser *et al.*, 1986). Strains of *B.avium* have a G + C mol percentage of 61.6- 62.6, whereas that for the other three *Bordetella* species lies within the range 67.7- 69.5 (Kerstens *et al.*, 1984). Sequences homologous to the *B.pertussis* *ptx* and *cya* operons were not identified in *B.avium* (Arico and Rappuoli, 1987; Brownlie *et al.*, 1988).

The fact that certain characteristics, but not others, are shared among the four *Bordetella* species (e.g. see Table 2.1), together with the emergence of recent molecular data, could elucidate the pathways by which the species have evolved. Arico *et al.* (1987) used a computer analysis of nucleotide sequence data for PT, and proposed that a single ancestral strain evolving from *B.pertussis* generated first the *B.pertussis* strain 18323, and then *B.parapertussis* and *B.bronchiseptica*. However, the presence

of an IS element in *B.pertussis* which is not present in *B.parapertussis* or *B.bronchiseptica* (McPheat and McNally, 1987b) suggests that *B.pertussis* is not the main branch of evolution.

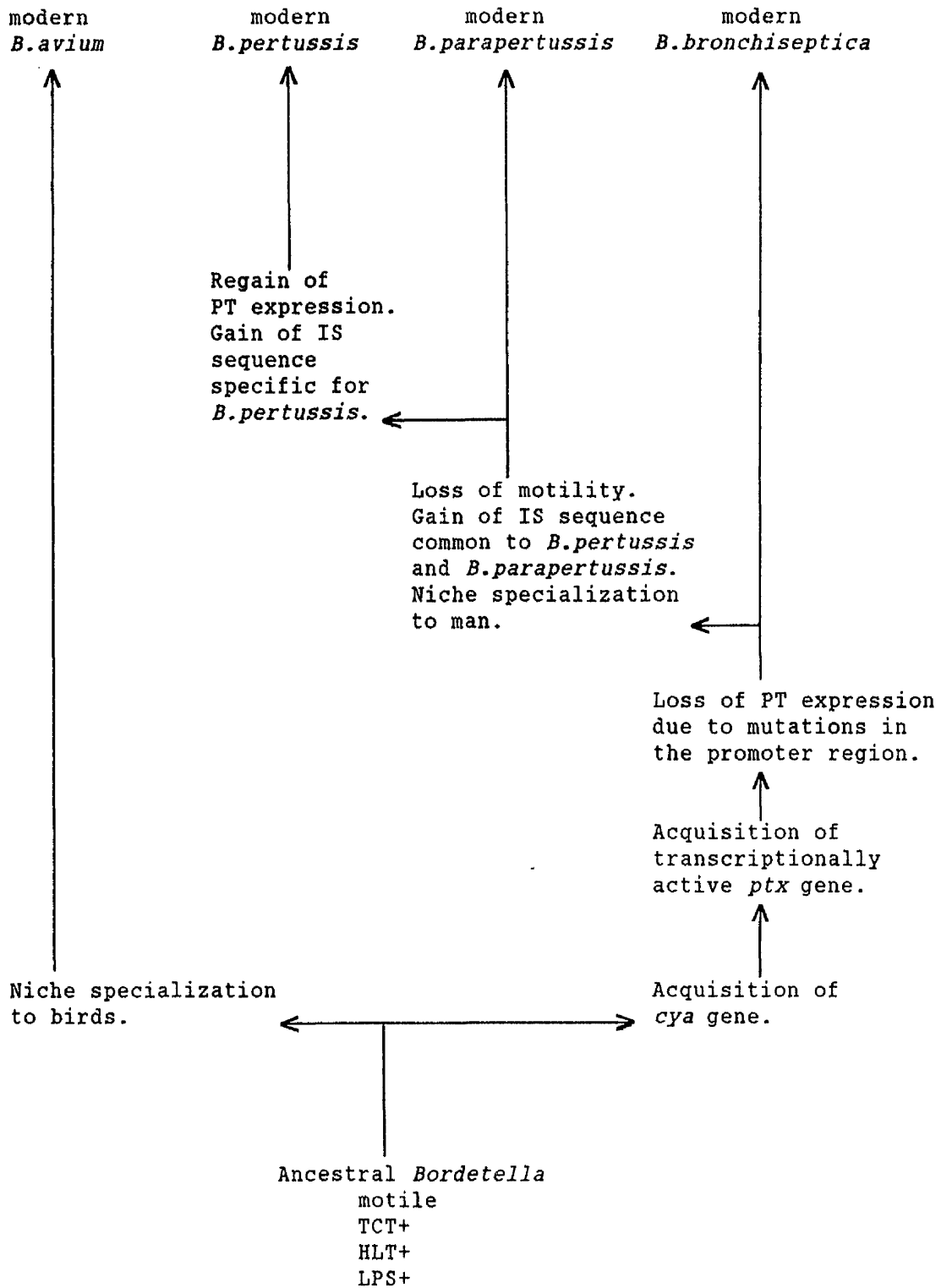
Wardlaw (1988) and Gross *et al.* (1989) have proposed schemes for pathways by which the genus *Bordetella* could have evolved; Figure 2.1 shows a possible phylogenetic tree of the genus, based on ideas from the aforementioned authors. Wardlaw (1988) and Gross *et al.* (1989) propose that the ancestral *Bordetella* strain was probably similar to present day *B.bronchiseptica* and, because of the distinctiveness of *B.avium*, there was probably early emergence of a line which lost AC expression and acquired an avian niche specialization. Alternatively, as proposed in Figure 2.1, the line leading to the mammalian bordetellae may have acquired the *cya* gene (perhaps by transposition from a eukaryotic source) after the divergence of *B.avium*.

Gross *et al.* (1989) propose that early on in the evolutionary process (after the divergence of *B.avium*) the ancestral *Bordetella* acquired transcriptionally silent *ptx* genes, and that later, a strain evolving towards *B.pertussis* acquired the ability to express PT. However, the fact that the mutations in *B.parapertussis* and *B.bronchiseptica* are concentrated within the promoter region (Arico *et al.*, 1987) may suggest the action of a selective pressure against PT expression in these species. Therefore, as proposed in Figure 2.1, the ancestral *Bordetella* may have acquired a transcriptionally active *ptx* gene, and production of PT may then have been selectively eliminated. *B.pertussis* and *B.parapertussis* share a number of features, suggesting the emergence of a line leading towards these species. However, the presence of common mutations in the *ptx* region of *B.parapertussis* and *B.bronchiseptica*, suggests that these mutational events

Figure 2.1.

Phylogenetic tree of the genus *Bordetella*.

Schematic representation of possible pathways by which modern *Bordetella* species have evolved from an ancestral strain. Based on ideas by Wardlaw (1988) and Gross *et al.* (1989).





occurred before the emergence of the line leading towards *B.pertussis* and *B.parapertussis*. *B.pertussis* may therefore have undergone a PT-transitional form, and later regained the ability to express PT through selection of promoter up mutations.

## Section 2.2. PERTUSSIS.

### 2.2.1. The clinical disease.

Pertussis (whooping cough) is an acute respiratory disease of world-wide distribution (see Fine, 1988) that affects mainly infants and young children. There are many descriptions of the classic disease (Lapin, 1943; Olson, 1975; Linnemann, 1979; Manclark and Cowell, 1984; Walker, 1988; Friedman, 1988).

The causative organism, *B.pertussis*, is transmitted from an infected person to a susceptible individual via respiratory droplets. The viable bacteria are inhaled, after which they presumably multiply on the respiratory tract mucosa. The incubation period can vary from 6 to 20 days (Lapin, 1943). Pertussis can be divided into three symptomatic stages: catarrhal, paroxysmal and convalescent. The catarrhal stage is often indistinguishable from that due to many other respiratory pathogens: symptoms resemble a common cold with rhinorrhoea, coryza, a mild cough, and occasionally a mild fever. After about 10 to 15 days, the severity and frequency of coughing episodes increases, which marks the onset of the paroxysmal stage (Olson, 1975). Fully developed paroxysms consist of groups of forceful coughs, leading to the eventual expectoration of tenacious mucus that has blocked the airway. The inspiration of air into the lungs

past a partially closed glottis results in the classical "whoop" associated with the disease (Olson, 1975; Manclark and Cowell, 1984). However, not all patients whoop, nor is there a whoop associated with every paroxysm (Wilson *et al.*, 1965; Zoumboulakis *et al.*, 1973). Vomiting and extreme exhaustion often follow a coughing episode. Many complications can occur during the paroxysmal stage, and of most concern are secondary infections, seizures, encephalopathy and death (Lapin, 1943; Olson, 1975; Linnemann, 1979; Manclark and Cowell, 1984). The convalescent stage is usually reached after about 4 weeks, although during this stage coughing paroxysms may occur sporadically, and secondary bacterial infections or other complications may arise (Olson, 1975; Miller and Fletcher, 1976; Linnemann, 1979).

There is evidence that if antibiotics are given during the first few days after exposure of a susceptible individual to *B.pertussis*, they may abort or shorten the duration of the disease (Linnemann *et al.*, 1975). *B.pertussis* is susceptible to erythromycin and, because it is safe in infants, this is usually the antibiotic of choice (Bass *et al.*, 1969; Islur *et al.*, 1975). However, once the disease is established, antibiotics are of limited value except perhaps in the treatment of secondary bacterial infections (MRC, 1953; Baraff *et al.*, 1978).

*B.pertussis* is believed to have no natural reservoir other than an infected person with clinical or subclinical disease (Kendrick, 1975). Although pertussis is considered to be predominantly a childhood disease, adults may be infected at a much higher rate than previously realized (Linnemann and Nasenbeny, 1977). It was recently shown that one quarter of adults with persistent cough were infected with *B.pertussis* (Robertson *et al.*, cited by Friedman, 1988). Adults with atypical disease may serve as a reservoir of infection (Linnemann, 1979).

### 2.2.2. Pertussis vaccines.

Control of pertussis relies on vaccine prophylaxis. For information on pertussis vaccines, see reviews by Lapin (1943), Anderson (1976), Manclark (1976), Miller *et al.* (1982), Wardlaw and Parton (1983b), Manclark and Cowell (1984), Robinson *et al.* (1985a), Griffiths (1988a,b) and Robinson and Ashworth (1988).

Attempts to immunize against pertussis started soon after the first isolation of *B.pertussis* by Bordet and Gengou (1906). Madsen (1933) was the first to describe the protective efficacy of whole-cell pertussis vaccine, during a pertussis epidemic in the Faroe Islands. Sauer (1933, 1937) obtained similar encouraging results. Clinical trials by Kendrick and Eldering (1936) demonstrated that pertussis vaccine protected against pertussis disease. Clinical trials in the 1940s and 1950s by the British Medical Research Council (MRC) validated the efficacy of pertussis vaccination, and demonstrated parallelism between the clinical efficacy and potency as measured by the ICMPT (MRC, 1951, 1956, 1959).

The whole-cell pertussis vaccines of today are produced from one or more strains of *B.pertussis*, killed and detoxified by various methods. They are normally combined with diphtheria and tetanus toxoids, and absorbed on an aluminium carrier (see Griffiths, 1988a). Procedures to standardize and control the manufacture of pertussis vaccine have been developed (recently reviewed by Cameron, 1988). The potency of pertussis vaccines is measured by the ICMPT (Kendrick *et al.*, 1947), and toxicity is controlled by the mouse weight-gain test (MWGT, Pittman and Cox, 1965). The bacterial content of pertussis vaccines is determined with an opacity standard developed by Perkins *et al.* (1973).

Pertussis vaccination is accompanied in many cases by adverse side

effects, ranging from mild to moderate transient reactions, to more severe reactions of a neurological nature which have been cause for major concern (Barkin and Pichichero, 1979; Cody *et al.*, 1981; Miller *et al.*, 1981). The reactions to whole-cell pertussis vaccine have been reviewed recently by Ross (1988).

Fears of reported side-effects to pertussis vaccination in the mid-1970s led to a marked reduction in vaccine uptake in the U.K., and an attendant increase in the incidence of pertussis (Jenkinson, 1978; Pollard, 1980; Griffith, 1981). Similarly, a dramatic increase in pertussis also occurred in Japan and Sweden after cessation of whole-cell pertussis immunizations in these countries (Sato *et al.*, 1984; Romanus *et al.*, 1987). These incidents substantiated the importance and efficacy of pertussis vaccines.

In the last 30 years there has been a considerable effort to develop an improved acellular pertussis vaccine, of lower toxicity than whole-cell pertussis vaccines, and with improved efficacy in preventing infection (see Griffiths, 1988b; Robinson and Ashworth, 1988). Pillemer (1954) made the first acellular vaccine using extracts of sonicated *B.pertussis* cells adsorbed on human erythrocyte membranes. Pertussis toxin, FHA and AGGs are immunogenic in humans (Ashworth *et al.*, 1983) and are the principle components considered for inclusion in pertussis acellular vaccines. Sato *et al.* (1984a) developed a component vaccine containing PT and FHA, which has been used for mass immunization in Japan since 1981. Two basic types of acellular vaccines are manufactured in Japan: a "Biken-type" which contains equal amounts of PT and FHA, and a "Takeda-type" which contains FHA, PT and AGG2 in the ratio 90:9:1. Vaccine trials have shown that these preparations are immunogenic and of low reactogenicity (see Griffiths, 1988b). Clinical

trials of two acellular vaccines, a monocomponent detoxified PT vaccine and a two-component detoxified PT and FHA vaccine, have been held in Sweden (reviewed by Griffiths, 1988b). Results showed that the efficacies of the vaccines were lower than anticipated, but a follow-up study has suggested that these acellular vaccines may be as effective as whole-cell vaccines (Olin *et al.*, 1989). A defined component vaccine, containing equal amounts of PT, FHA and AGGs (2 & 3), has been developed at CAMR (Robinson *et al.*, 1986). This vaccine has been tested in adult volunteers, and has been found to induce high antibody responses and to have low reactogenicity (see Robinson and Ashworth, 1988).

### 2.3. VIRULENCE-ASSOCIATED FACTORS PRODUCED BY *B.PERTUSSIS*.

With an ultimate aim of completely eradicating pertussis, development of a safe, efficacious vaccine relies increasingly on knowledge and understanding of individual bacterial components, their regulatory mechanisms, and modes of action in the pathogenesis of pertussis. During the last decade in particular, a voluminous literature has been produced on purification and molecular characterization of *B.pertussis* factors implicated in pathogenesis, as outlined in this Section.

Pertussis research is hampered by the lack of a suitable animal model which adequately mimics the disease process in humans. Much of the basic information about the pathogenesis of human pertussis has resulted from studies of mouse respiratory infection models. Animal models of pertussis have been reviewed recently by Sato and Sato (1988).

*B.pertussis* is a fastidious, slow-growing bacterium, initially thought

to have complex nutritional requirements. However studies have indicated that *B.pertussis* can grow in a relatively simple medium containing only a few amino acids, growth factors and salts (Hornibrook, 1939; Cohen and Wheeler, 1946; Jebb and Tomlinson, 1955; Stainer and Scholte, 1970). The sensitivity of *B.pertussis* to various inhibitors (see Rowatt, 1957a, b; Field and Parker, 1979a) adds to the difficulties of *in vitro* studies. However a major advancement in production of *B.pertussis* cultures is the finding by Imaizumi *et al.* (1983) that MeCD substantially promotes growth and PT production. The growth requirements of *B.pertussis* have been reviewed by Rowatt (1957b), Parker (1976) and Stainer (1988).

The use of purified *B.pertussis* components in both *in vitro* and *in vivo* systems allows characterization of their biological effects and speculation as to their possible roles in the pathogenesis of pertussis. Identification of antigens which induce a protective immune response should eventually lead to the isolation of peptides (or production of genetically-engineered or synthetic peptides) containing protective epitopes which could be included in future acellular vaccines of low toxicity.

Studies on the genetics of *B.pertussis* have greatly contributed to a better understanding of the mechanisms involved in pathogenesis of pertussis (as reviewed by Coote and Brownlie, 1988). A significant contribution to this area has been made by Weiss *et al.* (1983) who constructed a series of transposon Tn5 mutants of *B.pertussis* deficient in various putative virulence factors. A molecular approach to studying *B.pertussis* confers several advantages:

- i) *B.pertussis* DNA sequences can be moved to another (less fastidious) organism
- ii) structure and control of genes can be investigated

- iii) characterization of virulence factors can be simplified
- iv) production of proteins *in vitro* can be increased
- v) mutants can be constructed for evaluation of pathogenic effects and development of attenuated strains.

The pathogenesis of pertussis has 4 main stages: i) attachment of the bacteria to the respiratory tract, ii) growth of the organism and evasion of host defences which are directed at its elimination, iii) production of local disease effects and, iv) production of systemic disease by dissemination of toxins. *B.pertussis* produces an elaboration of factors implicated in pathogenesis, and some have been ascribed a particular role. For example, AGGs and FHA are most likely involved in adhesion of bacteria to the respiratory epithelium; TCT and HLT may contribute to the disease by causing local tissue damage; AC may assist survival of the pathogen by interfering with phagocyte function; PT may be largely responsible for the major systemic effects of pertussis. *B.pertussis* virulence factors have recently been reviewed by Wardlaw and Parton (1983a), Weiss and Hewlett (1986), Wardlaw and Parton (1988b) and Parton (1989). Certain characteristics of *B.pertussis* virulence-associated determinants (listed alphabetically) are detailed below.

#### 2.3.1. Adenylate cyclase / haemolysin.

The adenylate cyclase (AC) activity of *B.pertussis* exists in two forms: AC enzyme which possesses only enzymic AC activity, and AC toxin which has both enzymic activity and also the ability to enter mammalian cells and catalyze the formation of cyclic 3', 5' adenosine monophosphate (cAMP) from intracellular adenosine triphosphate (ATP). Production of haemolysin (HLY) is characteristic of virulent forms of *B.pertussis* (see Wardlaw and Parton,

1983a; Weiss and Hewlett, 1986).

Enzymic AC activity was first detected in commercial pertussis vaccine preparations by Wolff and Cook (1973). Hewlett and Wolff (1976) purified a 70 kD protein which possessed AC enzymic but no toxin activity, from the culture supernate of exponentially growing *B.pertussis*. Adenylate cyclase appears to be mainly extracytoplasmic in location (associated with either the periplasmic space or the extracellular surface of the cytoplasmic membrane), with a soluble fraction comprising up to 20% of the total AC activity being released into the culture medium (Hewlett *et al.*, 1976). *B.pertussis* AC exhibits the unusual feature of being activated, up to 1000-fold, by the eukaryotic calcium-binding protein, calmodulin (CaM, Wolff *et al.*, 1980). Although CaM stimulation of mammalian AC absolutely requires  $\text{Ca}^{2+}$  (Keller *et al.*, cited by Masure *et al.*, 1987), some authors have shown that AC activation by CaM is  $\text{Ca}^{2+}$ -independent (Greenlee *et al.*, 1982; Kilhoffer *et al.*, 1983). However, Hanski and Farfel (1985) reported that penetration of invasive AC absolutely requires  $\text{Ca}^{2+}$ . Modulated and avirulent phase variant strains do not express AC (Parton and Durham, 1978; Hewlett *et al.*, 1979; Wardlaw and Parton, 1979). The aforementioned properties of AC, plus the fact that it is produced by all mammalian *Bordetella* species (Endoh *et al.*, 1980), strongly implicate AC as a toxin and virulence factor.

The toxic activity of AC was first detected by Confer and Eaton (1982). Using crude urea extracts of *B.pertussis*, these authors demonstrated that AC traverses the cell membranes of human polymorphonuclear leukocytes and macrophages, is activated by CaM, and elicits an accumulation of intracellular cAMP. The elevation of intracellular cAMP levels results in a concomitant inhibition of phagocyte functions, such as zymosan stimulated



superoxide generation and the killing and ingestion of bacteria. These activities suggest a role for AC in *B.pertussis* pathogenesis in assisting survival of the pathogen by impairing host phagocyte defence mechanisms. Elevated intracellular cAMP levels as a result of *B.pertussis* AC activity have also been demonstrated for a number of mammalian cell types, including human lymphocytes, S49 murine lymphoma cells, turkey erythrocytes and rat oocytes, but not for human erythrocytes (Hanski and Farfel, 1985). Shattuck and Storm (1985) used partially purified and highly active AC preparations to confirm that AC was the component being internalized by eukaryotic cells and eliciting an increase in cAMP levels.

Initial steps to purify *B.pertussis* AC from culture supernates or from bacterial cell surface extracts, reveal that AC exists under a number of molecular forms, ranging from 43kD to 700kD (see Hanski and Farfel, 1985; Shattuck *et al.*, 1985; Kessin and Franke, 1986; Ladant *et al.*, 1986; Friedman, 1987; Masure *et al.*, 1987; Hewlett and Gordon, 1988). These variations may be partly due to the different methods of isolation used, and the sensitivity of AC to proteolysis. Rogel *et al.* (1988) found two forms of AC, a high (200kD) and a low (47kD) molecular weight species. Similarly, Masure and Storm (1989) purified proteins of 215kD and 45kD for AC. These authors propose that AC is synthesized as a precursor protein, and then proteolytically processed to the smaller form. Hewlett *et al.* (1985a) proposed that the AC toxin of *B.pertussis* consists of an enzymatically active (A) subunit and a receptor-binding (B) subunit, consistent with the A-B model for bacterial toxins.

The role of AC in *B.pertussis* pathogenesis was verified by Weiss *et al.* (1984) using a genetic approach. A series of Tn5 mutants constructed by Weiss *et al.* (1983) included several HLY deficient mutants which had

different levels of AC activity e.g. significant AC enzymic activity could be detected in BP349 (Hly-), but no AC activity could be detected in BP348 (Hly- Adc-). Transposon Tn5 is known to cause polar mutations (Berg *et al.*, 1980) and Weiss *et al.* (1983) suggested that the genes for AC and HLY were closely linked on the same operon, with the *cya* gene being located upstream of the *hly* gene. Several *B.pertussis* strains were tested for virulence in the infant mouse model: compared to wild-type *B.pertussis* strains, BP349 (Hly-) had reduced virulence and BP348 (Hly- Adc-) was avirulent (Weiss *et al.*, 1984). These findings demonstrated that HLY may play a role in *B.pertussis* pathogenesis, and that AC is an essential requirement for virulence of *B.pertussis*, at least in murine models.

Hewlett *et al.* (1985b) showed that extracts from wild-type *B.pertussis* strains, and from BP349 (Hly-) and BP357 (a Tn5 induced mutant deficient in PT production), were able to elicit a massive intracellular cAMP accumulation in S49 lymphoma cells. In contrast however, extracts from BP348 (Hly- Adc-) were without effect on cAMP levels in S49 lymphoma cells (Hewlett *et al.*, 1985). These data verified that *B.pertussis* AC is the component responsible for the large increase in intracellular cAMP levels seen in cells treated with *B.pertussis* extract.

Further weight was added to the importance of AC as a virulence factor by the finding that monoclonal Ab to AC was protective in an experimental mouse respiratory infection model (Brezin *et al.*, 1987). Adenylate cyclase is immunogenic in man: Farfel *et al.* (1990) have found that patients with pertussis, or subjects that have received pertussis vaccine, produce a high titre of anti-*B.pertussis* AC antibodies.

Brownlie *et al.* (1986) reported the isolation of a clone (pRMB1), from a gene library of *B.pertussis* TAB I DNA, which was able to restore AC and

HLY activities to BP348. Strain BP348 harbouring pRMB1 overproduced both HLY and AC, and regained its virulence capacity in murine models (Brownlie *et al.*, 1988). The Tn5 insertions in BP348 (Hly- Adc-) and BP349 (Hly-) were mapped to the same 10kb *Bam*HI fragment (Brownlie *et al.*, 1988).

Glaser *et al.* (1988a) used a recipient *cya*-defective *Escherichia coli* strain harbouring a plasmid which expressed high levels of a synthetic calmodulin, to identify <sup>cloned</sup> *B.pertussis* DNA which could complement the *cya* defect. Molecular analysis of clones revealed a 5.2kb open reading frame (ORF), with the potential to encode a protein of 1706 amino acids, which was assigned to the *B.pertussis cya* gene (Glaser *et al.*, 1988a).

Further studies by Glaser *et al.* (1988b) revealed that the 45kD secreted, calmodulin-~~responsive~~ AC of *B.pertussis* is synthesized as a 1706 amino acid bifunctional protein carrying both AC and HLY activities. These authors proposed the name 'cyclolysin' for the AC-HLY protein. Analysis of protein sequence data showed a significant degree of homology between the carboxy-terminal part of the AC precursor, *E.coli* alpha-haemolysin, and *Pasteurella haemolytica* leucotoxin (Glaser *et al.*, 1988b). Sequence analysis of a region downstream from the *cya* structural gene (*cyaA*) identified three ORFs: *cyaB*, *cyaD* and *cyaE*, coding for polypeptides of 712, 440 and 474 amino acid residues respectively. The gene products of *cyaB* and *cyaD* share homology with the gene products of *hlyB* and *hlyD*, known to be necessary for the transport of HLY across the cell envelope in *E.coli* (Glaser *et al.*, 1988b). These authors propose that *cyaA*, *cyaB*, *cyaD* and *cyaE* are organized in a single operon, and that the gene products of *cyaB*, *cyaD* and *cyaE* are necessary for secretion of the AC-HLY bifunctional protein across the cell membrane.

### 2.3.2. Agglutinogens.

A scheme for serotyping the genus *Bordetella*, based on the presence or absence of specific agglutinogens (AGGs) on the surface of the bacteria, was developed by Andersen (1953) and extended by Eldering *et al.* (1957). All strains of *B.pertussis* possess AGGs 1 and 7, and may also possess AGGs 2 to 6 in various combinations. Preston *et al.* (1982) proposed that AGGs 1, 2 and 3 are major, and AGGs 4, 5 and 6 are minor agglutinogens. Based on epidemiological data (Preston, 1963, 1965) the World Health Organization (WHO) recommended that whole-cell pertussis vaccines should include the three major AGGs, 1, 2 and 3 (WHO, 1979).

Ashworth and co-workers (1982) demonstrated that AGG 2 is a fimbrial protein, with a subunit molecular weight of around 22kD. These authors observed by electron microscopy that antibody to purified AGG 2 labelled fimbriae on serotype (ST) 1,2 organisms (Ashworth *et al.*, 1982, 1985). Serotype 2 fimbriae have also been purified by Zhang *et al.* (1985a) and Irons *et al.* (1985). Agglutinogen 3 has also been recognized as a fimbrial protein, with a slightly smaller molecular weight subunit than AGG 2 (Ashworth *et al.*, 1985; Irons *et al.*, 1985; Fredriksen *et al.*, 1988). The fimbrial nature of AGG 3 is a controversial issue: Carter and Preston (1984), Preston (1985) and Cowell *et al.* (1986) examined *B.pertussis* strains by electron microscopy and were unable to detect fimbriae on ST 1,3 strains, although strains containing AGG 2 or AGG 6 were fimbriated. These contradictory results may be explained by the different serotyping systems used. Strains identified as containing AGG 3 by the Preston serotyping system (Preston *et al.*, 1982) are also identified as containing AGG 6 when the Eldering serotyping system (Eldering *et al.*, 1957) is used (see Cowell *et al.*, 1987; Robinson *et al.*, 1989).

Cowell *et al.* (1986, 1987) purified fimbriae identified as AGG 6 on the basis of the agglutinating specificity of antisera raised against the purified protein. Antibodies to purified ST 2 and ST 6 fimbriae appeared to be monospecific based on agglutination tests of whole-cells, however a weak cross-reaction between ST 2 and ST 6 fimbriae was detected by ELISA (Cowell *et al.*, 1987). Irons *et al.* (1985), Robinson *et al.* (1985) and Zhang *et al.* (1985b) have also suggested that *B.pertussis* fimbriae may contain cross-reacting antigenic determinants. The ST 6 fimbriae purified by Cowell *et al.* (1986, 1987) and the ST 3 fimbriae purified by Irons *et al.* (1985) were both obtained from the same *B.pertussis* strain, and are therefore likely to be equivalent (Cowell *et al.*, 1986, 1987). Subsequent mention of AGG 3 in this thesis will refer to the fimbrial protein, as identified by the Preston serotyping system.

Ashworth *et al.* (1985) demonstrated the presence of both AGG 2 and AGG 3 on the surface of individual *B.pertussis* cells, indicating that the two antigenically distinct fimbriae can be simultaneously expressed. The helical structure of *B.pertussis* fimbriae has been described by Steven *et al.* (1986).

Fimbriae of other bacterial pathogens are involved in adherence (Isaacson, 1985) and by analogy, the fimbriae of *B.pertussis* may play a role in pathogenesis by mediating attachment of the bacteria to the ciliated respiratory epithelium. Antibody to fimbriae could be protective by blocking the initiation of the disease process: Gorringe *et al.* (1985) demonstrated that monoclonal antibodies to AGGs 2 and 3 inhibited binding of *B.pertussis* to Vero cells in a serotype specific manner. Robinson *et al.* (1985b) and Zhang *et al.* (1985b) found that mice immunized with purified fimbriae were protected against a subsequent aerosol challenge with

virulent *B.pertussis*.

The biochemical nature of the other AGGs has yet to be determined. It has been proposed that the polysaccharide chain of lipooligosaccharide has properties similar to AGG 1 (Li *et al.*, 1988; Robinson *et al.*, 1989). It has recently been suggested that AGGs 4, 5 and 6 may represent either minor epitopes associated with the main structural subunits or minor fimbrial proteins other than the repeating structural subunit (Robinson *et al.*, 1989).

The ST 2 fimbrial subunit gene (*fim2*) from *B.pertussis* has been cloned (Livey *et al.*, 1987). Analysis of the NH<sup>2</sup>-terminal amino acid sequence of purified ST 2 fimbriae from *B.pertussis* Tohama I enabled synthesis of an oligonucleotide probe for use in identifying ST 2 clones from a gene library of strain Wellcome 28 in pBR328. Nucleotide sequence analysis of the *fim2* gene revealed an ORF of 621 base pairs. Amino acids 27- 46 of the protein deduced from the nucleotide sequence matched the first 20 amino acids of the ST 2 fimbrial subunit determined by amino acid sequencing of the purified protein, indicating that the ST 2 subunit contains a 26 amino acid signal peptide. The molecular weight of the mature fimbrial subunit, calculated from the deduced amino acid composition, is 19.2kD. A region of homology to the -10 consensus sequence of *E.coli* promoters was identified, but homology to the -35 consensus sequence was absent, a feature frequently associated with promoters that are positively regulated. Livey *et al.* (1987) also analysed and compared the NH<sup>2</sup>-terminal amino acid sequences for the ST 2 and ST 3 subunits, and reported about 80% homology between the two, which agrees with findings by Cowell *et al.* (1987) and Mooi *et al.* (1987). A lesser degree of homology was found between the NH<sup>2</sup>-terminal *B.pertussis* fimbrial subunits, and those of *E.coli*, *Haemophilus influenzae*

and *Proteus mirabilis* (Livey et al., 1987; Mooi et al., 1987).

Mooi et al. (1987) hybridised a genomic blot of *SalI*-digested *B.pertussis* DNA, with a probe derived from the NH<sup>2</sup>-terminal region of the ST 2 fimbrial subunit. Three fragments were detected, suggesting the presence of three fimbrial subunit genes.

Using oligonucleotide probes derived from amino acid sequences of *B.pertussis* fimbrial subunits, Pedroni et al. (1988) identified a novel gene, designated *fimX*. Nucleotide sequence analysis of *fimX* identified an ORF of 628 base pairs. The deduced amino acid composition suggests that the mature *fimX* gene product, which has a predicted molecular weight of 20kD, is preceded by a signal peptide. The deduced NH<sup>2</sup>-terminal sequence of the mature *fimX* gene product is similar, but not identical, to the amino acid sequences of the ST 2 and ST 3 fimbrial subunits (Pedroni et al., 1988). There is evidence to suggest that *fimX* represents a silent gene: no third fimbrial subunit has been described for *B.pertussis*, and analysis of the promoter region has identified a deletion which would make expression of *fimX* unlikely (Willems et al., 1990).

The ST3 fimbrial subunit gene (*fim3*) has been cloned and sequenced (Mooi et al., 1990). Comparison with the *fim2* gene nucleotide sequence showed a conserved region (at position -20 to -50), upstream of the putative -10 box, which contained a stretch of 13- 15 C-residues. It was proposed that this C-rich region may be involved in serotype variation (Mooi et al., 1990).

Novotny et al. (1985a) identified a protective 68kD antigen (P.68) associated with virulent strains of *B.bronchiseptica*. With a monoclonal antibody to P.68, a homologous 69kD outer membrane protein (P.69) was found in *B.pertussis* (Novotny et al., 1985b). Purified P.69 protects mice from a

lethal ic *B.pertussis* challenge (Novotny *et al.*, 1985b). Brennan and co-workers (1988) identified P.69 as an agglutinin that reacts with the Eldering serotype 3 antiserum. The gene for P.69 has been cloned (Charles *et al.*, 1989). Computer analysis of the DNA sequence revealed an ORF capable of encoding a protein of around 93kD (P.93). The first 34 residues of the deduced amino acid sequence of P.93 have the features of a bacterial signal peptide, and other cleavage sites may also be present within the P.93 molecule (Charles *et al.*, 1989). The biochemical nature of P.69 remains to be determined.

#### 2.3.3. Filamentous haemagglutinin.

Keogh *et al.* (1947) first demonstrated that cultures of *B.pertussis* produce haemagglutinin, so named for its ability to agglutinate a variety of erythrocytes. *B.pertussis* produces three haemagglutinins: pertussis toxin, filamentous haemagglutinin (FHA) and an ornithine-containing lipid (Arai and Sato, 1976; Kawai *et al.*, 1982). The activity of FHA is characterized by its sensitivity to cholesterol inhibition (Sato *et al.*, 1983). By electron microscopy, FHA appears as fine filaments about 2nm in diameter and 40- 100nm in length (Arai and Sato, 1976; Morse and Morse, 1976). Originally it was thought that FHA was fimbrial in nature (Sato *et al.*, 1979; Morse and Morse, 1976), but Ashworth *et al.* (1982) demonstrated that FHA is a non-fimbrial protein.

Several authors have reported the purification of FHA from either static liquid or agar cultures (Arai and Sato, 1976; Arai and Munoz, 1979a; Irons and MacLennan, 1979; Irons *et al.*, 1983; Sato *et al.*, 1983). When examined by SDS-PAGE, the purified FHA protein consists of a heterologous



mixture of polypeptides, with molecular weights ranging from about 58 to 220 kD (Irons and MacLennan, 1979; Robinson *et al.*, 1981; Irons *et al.*, 1983). In a study using monoclonal antibodies to FHA, and the protease inhibitor PMSF, Irons *et al.* (1983) suggested that many of the lower molecular weight polypeptides are degradation products of the 220kD polypeptide.

Studies using murine models have indicated that FHA is a protective antigen (Robinson *et al.*, 1981; Sato *et al.*, 1979, 1981; Sato and Sato, 1984). Antibodies to FHA have been found to inhibit, or partially inhibit, adhesion of *B.pertussis* to various mammalian cells (Sato *et al.*, 1981; Gorringer *et al.*, 1985). The importance of FHA in mediating adherence to eukaryotic cells *in vitro* (Lenin *et al.*, 1986; Urisu *et al.*, 1986) suggests the role of adhesion for FHA in the pathogenesis of pertussis. *B.pertussis* Tn5 mutants BP353 (Fha-), BP354 (Fha-) and BP356 (Ptx-) showed a decreased ability to adhere to human ciliated cells *in vitro* (Tuomanen and Weiss, 1985). However the adherence ability of these mutants was restored by the addition of exogenous FHA or PT (Tuomanen *et al.*, 1985). Both FHA and PT are secreted into the medium during growth of virulent *B.pertussis* (Arai and Munoz, 1979b) and these components can be recaptured either by *B.pertussis* or unrelated bacteria, and still function as adhesins (Tuomanen, 1986). A model has been proposed for the mechanism of adherence of *B.pertussis* to human cilia, in which FHA and PT are secreted from virulent bacteria and each act as a bivalent bridge between the bacteria and one or more carbohydrate-containing receptors on the cilia of the respiratory epithelium (see Tuomanen, 1988).

There have been several reports on the cloning of the gene for FHA (Reiser *et al.*, 1985; Mattei *et al.*, 1986; Brown and Parker, 1987; Stibitz

*et al.*, 1988; Relman *et al.*, 1989).

Reiser *et al.* (1985) and Mattei *et al.* (1986) constructed genomic libraries of *B.pertussis* DNA, cloned in the  $\beta$ -galactosidase gene of bacteriophage  $\lambda$  gt11. Expression of immunologically-reactive fusion polypeptides in *E.coli* was detected using anti-FHA antiserum. Characterization of positive clones identified 0.7kb (Reiser *et al.*, 1985) and 2.9kb (Mattei *et al.*, 1986) regions of *B.pertussis* DNA containing coding sequence for a portion of FHA. Brown and Parker (1987) screened a genomic library of *B.pertussis* DNA cloned in the kanamycin resistance gene of cosmid pCP13, to identify clones expressing immunologically-reactive FHA (iFHA) in *E.coli*. The structural *fha* gene was mapped to a 6.5kb fragment, and its expression was under the control of the promoter of the kanamycin resistance gene of pCP13. The sizes of FHA polypeptides produced by *E.coli* were larger than those of *B.pertussis*, perhaps indicating that the *E.coli* product contains signal sequences which are removed by *B.pertussis* during secretion (Brown and Parker, 1987).

Using DNA probes constructed from chromosomal DNA surrounding the sites of Tn5 insertion in BP347 (Vir-) and BP353 (Fha-), Stibitz *et al.* (1988a) identified a cosmid clone (pUW21-26) which encompasses both the *vir* and *fha* loci. Stibitz *et al.* (1988a,b) further analysed pUW21-26 by Tn5 mutagenesis and identified three genetic loci for FHA: the structural gene (*fhaB*) and two loci involved in FHA expression (*fhaA* and *fhaC*). Analysis of the polypeptides encoded by a series of plasmids containing Tn5 mutations in the *fhaB* gene identified a putative ORF of approximately 6kb, with the capacity to encode a polypeptide of around 200kD. A 10kb *EcoRI* fragment adjacent to the *vir* locus encompasses the *fhaB* gene, which is transcribed in the opposite direction to *vir*. Tn5 insertions within the 4kb *fhaA* region

of pUW21-26 caused an overproduction of iFHA in *E.coli*. The Tn5 insertions in BP353 and BP354 were mapped to the *fhaA* locus (Stibitz *et al.*, 1988a,b). BP353 and BP354 were initially characterized as Pha- (Weiss *et al.*, 1983) but later shown to produce low levels of full-length FHA protein (Urisu *et al.*, 1986; Brown and Parker, 1987). Putative roles for FhaA and FhaC in regulating the synthesis and export of FHA have been suggested (Stibitz *et al.*, 1988a,b).

Relman *et al.* (1989) analysed the nucleotide sequence of the 10kb *EcoRI* fragment encompassing *fhaB* and identified an ORF of at least 9783bp with coding capacity for a protein of around 332kD. Putative cleavage sites have been identified within the deduced amino acid sequence. Two <sup>(RGD)</sup> tripeptide sequences containing arginine, glycine and aspartic acid, were identified as putative adherence factors: mutants deficient in this region were unable to bind to ciliated eukaryotic cells *in vitro*, strengthening the role for FHA in adherence.

RGD tripeptide sequences are normally involved in fibronectin binding.

#### 2.3.4. Heat-labile toxin.

Heat-labile toxin (HLT) has recently been reviewed by Nakase and Endoh (1988). Production of HLT by *B.pertussis* was first reported by Bordet and Gengou (1909). Subcutaneous injection of HLT in mice causes dermonecrotic lesions, splenic atrophy, and is lethal at high doses (see Cowell *et al.*, 1979; Sekiya *et al.*, 1982; Livey and Wardlaw, 1984). The toxic activity of HLT is destroyed by heating at 56°C for 10 min (Munoz, cited by Wardlaw and Parton, 1983a). Cowell *et al.* (1979) reported the cytoplasmic location of HLT, but Livey and Wardlaw (1984) suggested that HLT may also be partially exposed on the cell surface.

HLT has been purified by Onoue *et al.* (1963), Nakase *et al.* (1969), and more recently by Livey and Wardlaw (1984), Nakase and Endoh (1985), Zhang and Sekura (cited by Nakase and Endoh, 1988) and Endoh *et al.* (1986); the latter four reports give molecular weights of 89, 102, 130 and 102 kD respectively for the purified protein. Endoh *et al.* (1986) propose that the 102kD HLT protein comprises two 30kD and two 20kD subunit polypeptides. Purified HLT damages cell membrane permeability and has a vasoconstrictive action on vascular smooth muscle (Endoh *et al.*, 1988a, b). Nakase and Endoh (1988) have recently reviewed HLT.

#### 2.3.5. Lipopolysaccharide.

Like other Gram-negative bacteria, *B.pertussis* produces lipopolysaccharide (LPS, endotoxin) which has a characteristic range of activities in being heat-stable, antigenic, pyrogenic and toxic: *B.pertussis* LPS however has several unusual biochemical and biological features (as reviewed by Chaby and Caroff, 1988).

Chemical analysis of *B.pertussis* LPS has revealed that it may be composed of two distinct lipids (lipid A and lipid X) and two different oligosaccharide chains (types I and II) (Ayme *et al.*, 1980; Le Dur *et al.*, 1980; Moreau *et al.*, 1984). *B.pertussis* has two immunologically distinct LPS types (Le Dur *et al.*, 1980; Peppler, 1984). The two forms of LPS remain unchanged by phase variation and phenotypic modulation (Peppler and Schrumph, 1984).

*B.pertussis* LPS differs from other Gram-negative LPSs in its unique ability to induce resistance to mouse adenovirus infection (Winters *et al.*, 1985).

#### 2.3.6. Pertussis toxin.

Pertussis toxin (PT) has probably been the most extensively studied component of *B.pertussis*: its production, purification, molecular structure, enzymic activities and multiple biological effects *in vitro* and in experimental animals have recently been reviewed by Wardlaw and Parton (1983a), Sekura *et al.* (1985), Burns (1988), Furman *et al.* (1988), Irons and Gorringe (1988), Munoz (1988), Ui (1988), Gross *et al.* (1989) and Parton (1989).

Previous names for PT include histamine-sensitizing factor, lymphocytosis-promoting factor and islets-activating protein, referring to the diversity of its biological effects in experimental animals. Munoz and Bergman (1977) first proposed that these numerous biological effects were due to one component, pertussigen, subsequently designated pertussis toxin by Pittman (1979).

Pertussis toxin is a major virulence factor of *B.pertussis* and has been implicated as the component responsible for paroxysmal coughing in clinical pertussis (Pittman, 1979). A Tn5 mutant deficient in PT (BP357) had reduced virulence in a murine model, confirming the importance of PT as a virulence factor (Weiss *et al.*, 1984). Evidence for the role of PT in adherence has also been provided (Gorringe *et al.*, 1985; Tuomanen *et al.*, 1985). Wardlaw *et al.* (1976), Munoz *et al.* (1981), Sato *et al.* (1981; 1984a, b) and Granstrom *et al.* (1985) are among authors who have shown that PT is a protective antigen, either in animal models or in humans.

Early work on the purification of PT includes that of Arai and Sato (1976) who reported that, by electron microscopy, PT appears as spherical particles approximately 6nm in diameter. Subsequent reports on purification of PT include Tamura *et al.* (1982), Sato *et al.* (1983) and Sekura *et al.*

(1983). The toxin, which is released into the supernate by virulent *B.pertussis*, is a hexamer composed of five subunits, S1 to S5, in a molar ratio of 1:1:1:2:1 (Tamura *et al.*, 1982). Like diphtheria toxin, cholera toxin and *E.coli* heat-labile toxin, PT conforms to an A-B model (Gill, 1978): the A (active) moiety is composed of enzymically active subunit S1, and the B moiety comprises subunits S2 to S5, arranged in two heterodimers, (S2 + S4) and (S3 + S4), joined by S5 (Tamura *et al.*, 1982). The B moiety mediates binding of receptors on the surface of eukaryotic cells, and translocation of the toxic A moiety across the target cell membrane (Tamura *et al.*, 1983).

In the presence of NAD, the A moiety (S1) mediates ADP-ribosylation of a family of GTP-binding membrane proteins (G-proteins) that regulate enzymes involved in cellular metabolism, such as adenylate cyclase (AC), phospholipase C and cyclic GMP-phosphodiesterase (Katada and Ui, 1982; Katada *et al.*, 1983; Hsia *et al.*, 1984; Manning *et al.*, 1984; Van Dop *et al.*, 1984; and others, as reviewed by Ui, 1988). Two G-proteins are involved in the regulation of AC,  $G_s$  and  $G_i$ , which stimulate or inhibit AC respectively. Cholera toxin acts by ADP-ribosylating  $G_s$ , whereas  $G_i$  is the substrate for PT. The  $G_i$  protein receives signals from inhibitory receptors ( $R_i$ ) which in turn receive messages from inhibitory hormones. Pertussis toxin acts by uncoupling  $G_i$  from  $R_i$ , and the resulting effect is that cells are unable to inhibit AC activity. A number of different cell types are affected by PT, and the ensuing effects are numerous, as reviewed by Burns (1988) and Ui (1988). Lochter *et al.* (1989) showed that when S1 was mutated at tryptophan and glutamic acid residues (positions 26 and 129 respectively), the toxic activity of PT was abolished but the molecule retained its immunoprotective properties.

Cloning of PT has been reported by Locht *et al.* (1986), Locht and Keith (1986) and Nicosia *et al.* (1986). Nucleotide sequence analysis identified several ORFs clustered within a 3kb region, which code for PT subunits in the order S1, S2, S4, S5 and S3 (Locht and Keith, 1986; Nicosia *et al.*, 1986). The PT subunit genes have been designated *ptxA* (S1), *ptxB* (S2), *ptxC* (S3), *ptxD* (S4) and *ptxE* (S5) (Black and Falkow, 1987). The *ptxD* gene overlaps *ptxB* and *ptxE* (Nicosia *et al.*, 1986). The gene coding for S5 was tentatively identified on the basis of amino acid composition (Nicosia *et al.*, 1986) and later confirmed by immunological studies in which antisera raised against the cloned *ptxE* gene product recognized the native S5 subunit (Nicosia *et al.*, 1987). Each of the five subunit genes is preceded by a sequence encoding a putative signal peptide, suggesting that the PT subunits are secreted individually into the periplasm, where the holotoxin is assembled (Locht and Keith, 1986; Nicosia *et al.*, 1986). From the deduced amino acid sequences, the molecular weights of the mature peptides were calculated to be 26.0kD, 21.9kD, 21.9kD, 12.1kD and 11.0kD (Locht and Keith, 1986), or 26.2kD, 21.9kD, 21.9kD, 12.1kD and 10.9kD (Nicosia *et al.*, 1986), for subunits S1 to S5 respectively.

Subunits S2 and S3 share 75% nucleotide sequence homology and 70% amino acid homology, suggesting that they may have evolved by gene duplication (Locht and Keith, 1986; Nicosia *et al.*, 1986). The two subunits are immunologically cross-reactive (Nicosia *et al.*, 1987). However despite a high degree of homology, S2 and S3 cannot substitute for each other in the holotoxin (Tamura *et al.*, 1982). Amino-terminal homology was found between the S1 subunit of PT and the ADP-ribosyltransferase subunit A of cholera toxin (Locht and Keith, 1986; Nicosia *et al.*, 1986).

Sequences homologous to the consensus -10 and -35 regions of *E.coli*

promoters were identified only upstream of *ptxA*, indicating that the PT subunit genes are organized in a polycistronic operon. However, the putative -10 and -35 regions for *ptx* are separated by 21bp, not 17bp as required for efficient expression of *E.coli* promoters (Locht and Keith, 1986; Nicosia *et al.*, 1986). Nicosia and Rappuoli (1987) confirmed the location of the consensus -10 and -35 regions, and showed that transcription of the *ptx* operon starts 7bp downstream of the -10 region. A rho-independent transcription terminator was identified at the 3' end of *ptxC* (Locht and Keith, 1986; Nicosia *et al.*, 1986).

A sequence similar to the Shine-Dalgarno sequence of *E.coli* has been identified upstream of *ptxA* (Locht and Keith, 1986; Nicosia *et al.*, 1986). Lochter and Keith (1986) identified a similar putative translational sequence in front of the *ptxD*, *ptxE* and *ptxC* genes; Nicosia *et al.* (1986) identified a new consensus sequence in a position corresponding to that of a ribosome binding site, preceeding the *ptxB*, *ptxD*, *ptxE* and *ptxC* genes.

The Tn5 insertions in mutants BP356 (Ptx-) and BP357 (Ptx-) (Weiss *et al.*, 1983) have been mapped to *ptxC* (Locht *et al.*, 1986; Lochter and Keith, 1986; Nicosia *et al.*, 1986). These mutants are unable to produce active PT in culture supernates (Weiss *et al.*, 1983) however preliminary data indicates that subunits S1, S2, S4 and S5 can be detected in cells (Marchitto *et al.*, 1987b; Nicosia and Rappuoli, 1987). These data suggest that the S3 subunit is required for assembly and release of PT into the culture medium.

Black and Falkow (1987) used allelic exchange to introduce a number of defined mutations into the *ptx* region of *B.pertussis*. Examination of PT gene expression suggested that, i) an intact S1 subunit gene is required for synthesis of S2 and S4, indicating that *ptxA*, *ptxB* and *ptxD* may form a



single transcriptional unit, ii) mutations in *ptxE* adversely affect the synthesis or stability of S1, and iii) mutations in *ptxD* affect synthesis of S2.

#### 2.3.7. Tracheal cytotoxin.

Tracheal cytotoxin (TCT) is the most recently identified toxin produced by *B.pertussis* (Goldman *et al.*, 1982). Purified TCT causes ciliostasis and specific damage to ciliated cells in hamster tracheal ring cultures, demonstrating a possible role for TCT in respiratory tract pathology (Goldman *et al.*, 1982). Cultured hamster tracheal epithelial cells exposed to *B.pertussis* supernates show an inhibition of DNA synthesis, implying that TCT may have a toxic effect for the basal cells in the respiratory epithelium (Goldman, 1986).

Tracheal cytotoxin has been purified and characterized (Goldman *et al.*, 1982; Goldman and Herwaldt, 1985; Goldman, 1986). Diaminopimelic acid and muramic acid are constituents of bacterial peptidoglycan: these components are also found in TCT, indicating that TCT is derived from peptidoglycan. Rosenthal *et al.* (1987) identified as TCT, soluble peptidoglycan fragments released by *B.pertussis* during broth culture. The structure of TCT suggests that it does not represent a peptidoglycan precursor form, but is likely to be released as a result of hydrolysis of peptidoglycan fragments from intact bacterial peptidoglycan (Rosenthal *et al.*, 1987).

Production of TCT does not appear to be affected by phase variation (Goldman, 1986). Further information on TCT can be found in a review by Goldman (1988).

#### 2.3.8. X-mode specific envelope polypeptides.

Analysis of *B.pertussis* cell envelope preparations by SDS-PAGE identified two major polypeptides associated with virulent (X-mode) strains (Parton and Wardlaw, 1975). These 28kD and 30kD X-mode specific envelope polypeptides (X-OMPs) were absent from profiles of phase IV *B.pertussis* strains, and strains which had been grown under modulating conditions (Parton and Wardlaw, 1975; Wardlaw *et al.*, 1976; Wardlaw and Parton, 1979). The X-OMPs as yet remain uncharacterized.

### Section 2.4. REGULATION OF EXPRESSION OF VIRULENCE DETERMINANTS IN *B.PERTUSSIS*.

As described above, virulent *B.pertussis* strains produce multiple virulence determinants necessary for the organism to establish infection and produce disease effects. The expression of these virulent-phase genes is influenced by both phenotypic and genotypic changes, in processes called phenotypic modulation and phase variation respectively. In addition, *B.pertussis* strains can exhibit heterogeneity in serotype (serotype variation). These phenomena are described below, and have been reviewed by Robinson *et al.* (1986b) and Coote and Brownlie (1988).

#### 2.4.1. Phenotypic modulation.

In response to certain environmental stimuli, such as low temperature (<28°C) or high levels of certain salts or organic acids, *B.pertussis* strains exhibit a freely reversible, phenotypic change called phenotypic (or antigenic) modulation (Lacey, 1960; Pusztai and Joo, 1967). Growth of

*B. pertussis* on normal medium containing NaCl produces X-mode or virulent cells, however growth with high levels of MgSO<sub>4</sub> (or other inorganic or organic salts, or low temperatures) gives rise to antigenically-distinct C-mode or avirulent cells (Lacey, 1960). An intermediate mode (I-mode) was also described by Lacey (1960), but this may have represented a population containing both X-mode and C-mode cells, rather than individual cells being I-mode (see Robinson *et al.*, 1986b). Virulent properties can be readily restored to modulated cells by culture on appropriate medium (Lacey, 1960). A high concentration of nicotinic acid in the growth medium induces a similar phenotypic modulation (Pustzai and Joo, 1967). In response to modulation signals, expression of certain genes is repressed. Virulence-associated factors lost during modulation include:-

AC (Parton and Durham, 1978; Hall *et al.*, 1982; McPheat *et al.*, 1983; Brownlie *et al.*, 1985a)

AGGs (Holt and Spasojevic, 1968; McPheat *et al.*, 1983)

FHA (Lacey, 1960)

HLT (Livey *et al.*, 1978; Idigbe *et al.*, 1981)

HLY (Lacey, 1960)

PT (Wardlaw *et al.*, 1976; Idigbe *et al.*, 1981; Robinson *et al.*, 1983; Brownlie *et al.*, 1985a)

X-OMPs (Wardlaw *et al.*, 1976; Idigbe *et al.*, 1981; Brownlie *et al.*, 1985a).

Additional factors not expressed in C-mode cells include cytochrome d (Dodrogosz *et al.*, 1979; Ezzell *et al.*, 1981b). Hydrophobicity of *B. pertussis* cells is affected by modulation: X-mode cells are hydrophobic whereas C-mode cells are hydrophilic (Robinson *et al.*, 1983). Modulated cells have a reduced adherence ability (Burns and Freer, 1982; Robinson *et al.*, 1983; Gorringer *et al.*, 1985; Redhead, 1985). For many bacterial

species, virulent strains can be identified by their ability to absorb certain dyes, such as Congo red (CR, Payne and Finkelstein, 1977). Parton (1988) found that modulated *B.pertussis* cells exhibit a CR- phenotype, whereas non-modulated cells are CR+. Lacey (1960) described a group of antigens which were expressed only in C-mode cells. Calderwood *et al.* (1988) and Knapp and Mekalanos (1988) have identified a number of genes whose expression is activated by modulators. Brownlie *et al.* (1985a) demonstrated that the  $\text{SO}_4^{2-}$  anion was the important component of  $\text{MgSO}_4$  for inducing modulation. Melton and Weiss (1989) examined the effect of a number of cations and anions on modulation, and found that only  $\text{SO}_4^{2-}$  anions eliminated transcription of virulence-associated genes.

$\text{MgSO}_4$ -induced and nicotinic acid-induced modulation are distinct: the latter gives a different serological response, and cells retain HLT expression (Pusztai and Joo, 1967). McPheat *et al.* (1983) found that nicotinic acid has a modulating effect on AGGs 2 and 3, but causes an increase in production of AGG 1. Growth with high concentrations of nicotinamide has no modulating effect on *B.pertussis*, suggesting that the carboxyl group may be an important mediator of modulation (Wardlaw *et al.*, 1976). Schneider and Parker (1982) showed that modulation could be induced by growth with two analogues of nicotinic acid, but not by growth with nicotinic acid containing a modified carboxyl group. The fact that  $\text{MgSO}_4$ -induced and nicotinic-acid induced modulation are distinct, suggests that they mediate their effect via different intermolecular interactions.

Brownlie *et al.* (1985a) studied the effect of several inorganic and organic salts on production of AC, PT and X-OMPs, and found that the loss of these three properties occurred concomitantly during modulation. This added weight to the hypothesis of Wardlaw and Parton (1979) that expression

of virulence determinants in *B.pertussis* is controlled by a common regulatory mechanism.

Adenylate cyclase plays a role in the regulation of expression of certain proteins in prokaryotes, including virulence-associated proteins of *E.coli* (Eisenstein *et al.*, 1981; Martinez-Cadena *et al.*, 1981). By analogy, a putative regulatory role was postulated for *B.pertussis* AC (Parton and Durham, 1978; Hewlett *et al.*, 1979; Wardlaw and Parton, 1979). This theory was negated by Brownlie *et al.* (1985b) who found that AC did not have a causal effect in the loss of *B.pertussis* virulence factors, but that AC was itself influenced by the same regulatory mechanism perturbed during modulation.

Generally, studies have indicated that the rate of loss of virulence components during modulation correlates closely with the theoretical values obtained, assuming complete repression of synthesis of these components on exposure to the modulator (Hall *et al.*, 1982; Robinson *et al.*, 1983; Brownlie *et al.*, 1985b). However studies by Idigbe *et al.* (1981) and Robinson *et al.* (1983), on MgSO<sub>4</sub> - and nicotinic acid-induced modulation respectively, suggested that the loss of virulence components occurred at a higher rate than could be accounted for by a simple growth-dilution effect. These authors proposed that a selective-destruction of X-mode components may occur during modulation. It is now known that modulation acts at the transcriptional level (Gross and Rappuoli, 1989; Melton and Weiss, 1989). Gross and Rappuoli (1989) studied the kinetics of the change in transcription during modulation in *B.pertussis*. These authors found that the addition of MgSO<sub>4</sub> to the growth medium caused an immediate stopping of the *ptx* operon, which resulted in the disappearance of mRNA from the cells within 10 minutes. However, resumption of transcription when cells were

subsequently shifted to permissive conditions, was slow.

Phenotypic modulation also occurs *in vivo* (Lacey, 1960). At the late stages of infection, modulation could confer several advantages:

- i) evasion of the immune response
- ii) conservation of metabolic energy (synthesis of virulence-associated factors is no longer required)
- iii) the change in hydrophobicity may assist expulsion of the organism, and transfer to a new host.

#### 2.4.2. Phase variation.

Phase variation was originally described by Leslie and Gardner (1931) as a progression of antigenic changes through four distinct phases, I, II, III and IV, which occurred when *B.pertussis* was repeatedly subcultured *in vitro*. Phases I and II were toxic to guinea-pigs whereas phases III and IV were not. However, Lawson (1939), Flosdorf *et al.* (1941) and Standfast (1951) proposed that phase variation was a more complex process, which involved many intermediate forms. In contrast to the view of Leslie and Gardner (1931) that the process of phase variation was an ordered step-wise process, Standfast (1951) proposed that many random changes occurred during phase variation. Phase variation may also occur *in vivo*: Kasuga *et al.* (1954b) found that organisms isolated from patients in the early stages of infection were phase I, whereas those isolated in the later stages were avirulent phase variants. The change in antigenicity could therefore represent a defence mechanism for the organism to evade immune detection.

Although reference antisera is no longer available for identification of the phases described by Leslie and Gardner (1931), the terms phase I and

phase IV are still used to describe virulent strains (which have the potential to synthesize virulence-associated factors) and avirulent strains (which are unable to express virulence-associated factors) respectively. Other terminology used to describe the phase change includes; phase I to phase III (Kasuga *et al.*, 1954a), fresh isolate to degraded state (Parker, 1979) and domed/haemolytic to flat/non-haemolytic (Peppler, 1982).

Avirulent phase variant strains are deficient in several components, including AC, AGGs, HLT, HLY, PT, certain outer membrane proteins and cytochrome d (Wardlaw *et al.*, 1976; Parton and Durham, 1978; Dobrogosz *et al.*, 1979; Wardlaw and Parton, 1979; Ezzell *et al.*, 1981b; Peppler, 1982). Avirulent strains have an increased resistance to certain inhibitory substances and a number of antibiotics (Parker, 1976; Dobrogosz *et al.*, 1979; Bannatyne and Cheung, 1984; Peppler and Schrupf, 1984a). The ability to grow on nutrient agar is characteristic of phase variant strains (Leslie and Gardner, 1931; Field and Parker, 1979b; Peppler and Schrupf, 1984a). The colony morphology of phase variants is flat, whereas that of virulent strains is domed (Peppler, 1982). Phase variant *B.pertussis* strains are unable to bind Congo red (Parton, 1988). As yet there is no evidence for the existence of genes which are expressed only in avirulent phase variant strains.

Parker (1976, 1979) suggested that phase variation is a multi-step, non-ordered process in which an accumulation of random mutations, selected by *in vitro* cultivation, finally leads to the avirulent phase. Goldman *et al.* (1984) selected *B.pertussis* phase variants by growth on Stainer and Scholte (SS) agar. Variants were screened for HLY, PT and FHA. Four different phenotypic classes were obtained; Hly+Ptx+Fha+ (7-11%), Hly-Ptx+Fha+ (17%), Hly-Ptx-Fha+ (5-11%) and Hly-Ptx-Fha- (65%). The

identification of only four phenotypes out of eight possible permutations, led Goldman *et al.* (1984) to propose that during phase variation, virulence factors are lost in an ordered way.

Various workers have studied the frequency at which phase variation occurs for *B.pertussis*. Peppler (1982) and Goldman *et al.* (1984) selected phase variants by growth on SS-agar and observed a frequency of variation of around  $10^{-6}$ . Weiss and Falkow (1984) used growth on BG-agar containing erythromycin to select Hly<sup>-</sup> colonies. These variants, which also lacked FHA and PT, arose at a frequency of  $10^{-3}$  to  $10^{-6}$ , depending on the strain.

During continual subculture, Weiss and Falkow (1984) observed that spontaneous avirulent phase variants reverted back to virulent phase at a low frequency. The reversion may have been influenced by environmental factors present in certain batches of media. Weiss and Falkow (1984) demonstrated two complete cycles of phase variation, indicating that the process is a reversible single-step event. This eliminated the possibility that phase variation involved loss of a plasmid or prophage which encoded the virulent-phase genes.

#### 2.4.3. Regulation by *vir* of expression of virulent-phase genes.

The simultaneous loss of virulence-associated factors during phenotypic modulation and phase variation led Wardlaw and Parton (1979) to hypothesize that a common regulatory mechanism controlled expression of virulent-phase genes in *B.pertussis*. Genetic evidence for this was provided by Weiss *et al.* (1983).

A vector system suitable for delivery of transposons into the *B.pertussis* chromosome has been described (Weiss and Falkow, 1983a). A



chimeric plasmid, pUW964, containing broad host-range conjugation genes from RK2, a ColE1 origin of replication, and Tn5, was constructed by Weiss *et al.* (1983). This "suicide" plasmid was transferred by conjugation from *E.coli* to *B.pertussis* BP338 (a nalidixic acid derivative of strain Tohama I). Due to the limited host-range for ColE1 incompatibility group plasmids, pUW964 was unable to replicate in *B.pertussis*. Mutants containing a chromosomal Tn5 insertion were selected by kanamycin resistance conferred by Tn5, and screened for loss of virulence-associated factors (Weiss *et al.*, 1983).

A Tn5 mutant was obtained (BP347) which was Hly-, and when further characterized was found to be deficient in PT, AC, FHA and HLT. A later study identified the Tn5 mutant BP359, which also had a pleiotropically-negative phenotype for expression of virulence-associated determinants (Weiss and Falkow, 1984). The Vir- phenotype of both mutants was due to a single Tn5 insertion in each case, which verified the hypothesis that a single genetic region is required for the expression of virulence-associated genes (Weiss and Falkow, 1984).

The series of Tn5 mutants isolated by Weiss *et al.* (1983) included BP348 (Hly-Adc-), BP349 (Hly-), BP353 (Fha-), BP354 (Fha-), BP356 (Ptx-) and BP357 (Ptx-). These mutants enabled the pathogenic effects of the individual factors to be evaluated, as described in Section 2.3. Hybridisation studies with a Tn5-specific probe showed that each mutant contained only a single copy of Tn5, which mapped to a different *EcoRI* fragment for each class of mutant (Weiss *et al.*, 1983). This provided genetic evidence that the virulence genes (apart from *hly* and *cya*, as described in Section 2.3.1) were not physically linked. The possibility that virulence genes were arranged in a polycistronic operon with their

expression being controlled by a single promoter was therefore eliminated.

Weiss and Falkow (1984) proposed a model for the co-ordinate regulation of virulence determinants in *B.pertussis* (see Figure 2.2). These authors proposed that a virulence regulatory locus, *vir*, which was inactivated by the Tn5 insertions in BP347 and BP359, encodes a *trans*-acting positive effector that positively induces the expression of virulent-phase genes. It was postulated that the effector could be the protein encoded by *vir* (Vir), or a product generated by the activity of Vir. In response to modulating conditions, or as a result of the genotypic changes produced by phase variation, the *vir* gene product would no longer be produced, and synthesis of virulence-associated factors would be repressed.

Regulation of virulence in *B.pertussis* fits into a common theme described for other bacteria e.g. *V.cholerae* and *E.coli*, whereby the co-ordinate transcriptional regulation of multiple virulence determinants is controlled by a central regulatory locus (see Calderwood *et al.*, 1988). Not all *B.pertussis* virulence-associated factors are regulated by *vir*: neither LPS nor TCT are affected by phase variation (Peppler, 1984; Goldman, 1986).

The *vir* locus of *B.pertussis* has recently been cloned (Stibitz *et al.*, 1988a). Two recombinant cosmid clones (pUW21-2 and pUW21-26) were identified which encompassed both the *vir* and *fha* loci. Expression of iFHA was detected in *E.coli* containing pUW21-2 or pUW21-26, but not in *E.coli* containing cosmids encoding *vir* alone or *fha* alone, confirming the requirement of *vir* for FHA expression. The synthesis of iFHA in *E.coli* was repressed in response to modulation signals (Stibitz *et al.*, 1988a).

Tn5 mutagenesis of pUW21-26 in *E.coli* defined a 5kb region for *vir*, close to the *fhaB* gene. This region was required for expression of iFHA, and encompassed the sites of Tn5 insertion in Vir- mutants BP347 and BP359.

Figure 2.2.

**Model for *vir*-regulated gene expression in *B.pertussis*.**

The schematic representation of the co-ordinate regulation of virulent-phase genes is based on information from Weiss and Falkow (1984) and Knapp and Mekalanos (1988).

Under normal conditions the virulence regulatory gene (*vir*) is transcribed and produces a *trans*-acting positive effector (Vir). Production of Vir induces expression (perhaps by binding to a promoter region and initiating transcription) of *vir*-activated genes (*vag* loci), and represses *vir*-repressed genes (*vrg* loci).

In response to modulating conditions, Vir is no longer produced: expression of *vag*-loci is repressed, and expression of *vrg*-loci is activated. The *mod* gene product could act as an environmental sensor which controls the expression of *vir*.

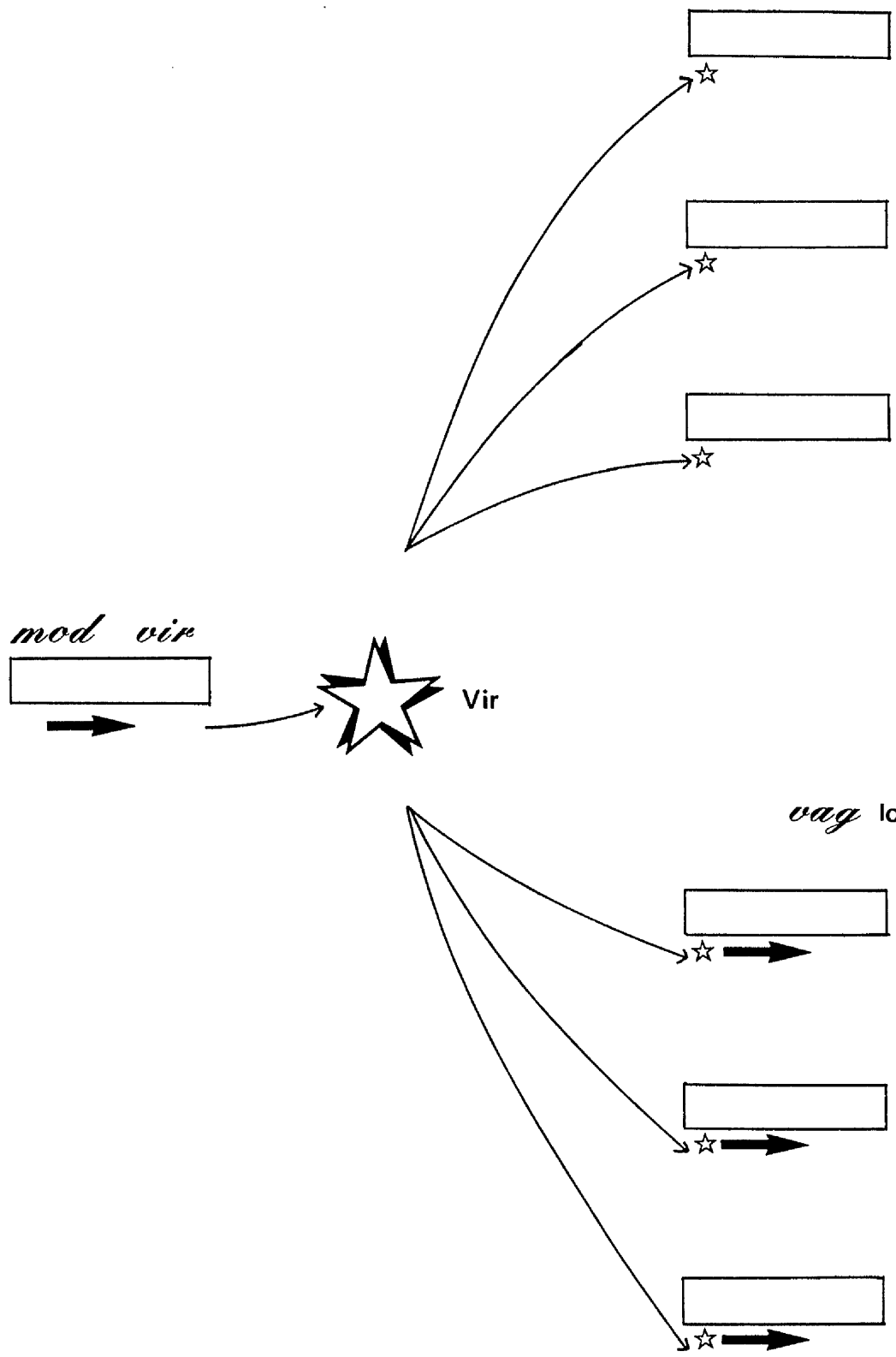
Similarly, as a result of mutations in the *vir* region, phase variant strains would no longer produce Vir, and *vag*-loci would no longer be expressed.

*org* loci

*mod vir*

Vir

*vag* loci



A Tn5 insertion between *vir* and *fhaB* did not abolish synthesis of iFHA, indicating that *vir* and *fhaB* represent separate transcriptional units (Stibitz *et al.*, 1988a).

Knapp and Mekalanos (1988) used Tn*phoA* to generate gene fusions in *B.pertussis* strain 18323. The PhoA activity of secreted hybrid proteins was determined in the presence and absence of modulators. Two sets of genes were identified: *vir*-activated genes (*vag* loci) whose expression requires the *vir* locus, and *vir*-repressed genes (*vrg* loci) which are not expressed under normal growth conditions (see Figure 2.2). Growth in the presence of modulators represses expression of *vag* loci, and derepresses expression of *vrg* loci. When a *vag*::Tn*phoA* gene fusion was converted to a *cat* transcriptional fusion, CAT activity was reduced in response to modulators, suggesting that modulation occurs at the transcriptional level in *B.pertussis*. The functions of *vrg* genes are presently unknown.

Knapp and Mekalanos (1988) isolated spontaneous mutants which constitutively expressed *vag* loci, even in the presence of modulators. The mutated region was designated *mod*. These *mod* mutations also resulted in prevention of derepression of *vrg* loci by modulators. It was possible that the *mod* constitutive mutations represented a new class of mutations in *vir*. Characterization of one *mod* mutant showed that the *mod* and *vir* genes were closely linked, but distinct, when *vir* was defined as a 2.6kb *EcoRI* fragment. However, further analysis is necessary to determine the exact nature and location of these *mod* mutations in the *vir* region. Knapp and Mekalanos (1988) proposed that the *vir* and *mod* gene products interact at some level during the control of *vir*-regulated gene expression, and suggested that the *mod* gene product may act as an environmental sensor required for the expression of *vir*.

Phase variation can be explained by loss of expression of *vir* (Weiss and Falkow, 1984). A Vir+ phenotype was restored to avirulent phase variant strains when the *B.pertussis* *vir* locus was provided in-*trans* (Brownlie *et al.*, 1988; Stibitz *et al.*, 1988a). This demonstrated that the genomic change which occurred during phase variation was contained within *vir*. Phase variation in bacteria can be caused by DNA rearrangements. In *Salmonella typhimurium*, the expression of two types of flagellar antigen is controlled by a DNA rearrangement which involves inversion of a DNA sequence (Zeig *et al.*, 1977). Similarly, pilus expression in *Neisseria gonorrhoeae* is also caused by a DNA rearrangement (Meyer *et al.*, 1982). Control of expression of *vir* by an invertible DNA sequence was proposed as a mechanism of phase variation in *B.pertussis* (Weiss and Falkow, 1984). Sequences homologous to the DNA inversion genes found in strains of *E.coli* and *S.typhimurium* have been detected in *B.pertussis*, suggesting that DNA rearrangement by a DNA invertase could represent one mechanism of phase variation in *B.pertussis* (Foxall *et al.*, 1990).

Lax (1985) observed that the frequency of phase variation for *Bordetella* was similar to the frequency expected for the random mutation of a bacterial gene, and proposed that phase variation may result from a random mutation in the *vir* locus followed by *in vitro* selection of phase variants. Goldman *et al.* (1987) found that DNA from avirulent phase variant strains was resistant to digestion by certain restriction endonucleases, suggesting that a DNA modification, perhaps methylation, was associated with phase change.

Stibitz *et al.* (1989) cloned the *vir* locus from a series of Vir+ and Vir- *B.pertussis* Tohama strains, derived one from the other by phase variation. The *vir* loci cloned from virulent phase strains were able to

confer a Vir<sup>+</sup> phenotype to avirulent phase variant strains, whereas the *vir* loci cloned from Vir<sup>-</sup> strains were unable to restore a Vir<sup>+</sup> phenotype. By recombination between deletion derivatives of a *vir*<sup>+</sup> allele and chromosomal *vir*<sup>-</sup> alleles in avirulent phase variant strains, the difference between the *vir*<sup>+</sup> and *vir*<sup>-</sup> form of the *vir* locus was mapped to a 1.4kb *Sall* fragment. Preliminary sequence data of this region revealed a single frame-shift mutation. In one position, the *vir*<sup>+</sup> form had a run of six G residues, whereas the *vir*<sup>-</sup> form had a run of seven Gs. These data suggested that, at least for strain Tohama, one mechanism of phase variation appears to be due to a frame-shift mutation within *vir*.

Nucleotide sequence analysis of the *vir* region has identified three tandemly arranged genes, and the predicted products of two of these genes share extensive homology with a family of two-component regulatory proteins (Arico *et al.*, 1989). A number of studies have recently demonstrated that expression of *vag*-loci is positively controlled at the level of transcription (Nicosia and Rappuoli, 1987; Knapp and Mekalanos, 1988; Melton and Weiss, 1989; Miller *et al.*, 1989; Roy *et al.*, 1989). One of the *vir* gene products is a transcriptional activator (Arico *et al.*, 1989; Miller *et al.*, 1989; Roy *et al.*, 1989, 1990). More detailed information on the molecular analysis of *vir* is given in Section 5.1.6.

Analysis of the *ptx* promoter region has shown that in addition to the *trans*-acting factor encoded by *vir*, efficient transcription of the *ptx* operon requires a region of 170bp upstream of the transcriptional start site (Gross and Rappuoli, 1988). Within this region, in addition to the sequences homologous to the consensus -35 and -10 regions of *E.coli* promoters, at least two features are essential for *ptx* promoter activity: a 21bp direct repeated sequence from position -157 to -117 (which may

represent the binding site for the *trans*-acting factor), and a stretch of 4 C-residues from position -34 to -31 (Gross and Rappuoli, 1988, 1989). A similar stretch of C-residues is also found in the promoter regions of the *fim* genes (Livey *et al.*, 1987; Mooi *et al.*, 1990; Willems *et al.*, 1990).

#### 2.4.4. Serotype variation.

Like other *vag*-loci, the *B.pertussis* *fim2* and *fim3* genes are positively regulated by *vir* at the level of transcription, but in addition, expression of these genes is controlled at an individual level in a process called serotype variation, or fimbrial phase variation (Willems *et al.*, 1990). Stanbridge and Preston (1974a) serially subcultured single colonies of defined serotype, and detected variants which had independently lost or gained expression of the fimbrial AGGs 2 or 3, although AGG 1 (non-fimbrial) was always expressed. A particular *B.pertussis* strain may produce no fimbriae (ST 1), only one type of fimbriae (ST 1,2 or 1,3), or both types of fimbriae (ST 1,2,3). A frequency of  $10^{-3}$  to  $10^{-4}$  has been reported for serotype variation (Stanbridge and Preston, 1974a). This phenomenon occurs *in vivo* in experimental animals (Stanbridge and Preston, 1974b; Preston and Stanbridge, 1976; Preston *et al.*, 1980) and in the child (Preston and Stanbridge, 1972). The serotype variation in *B.pertussis* may be analogous to the rapid changes in fimbriae which have been reported for *E.coli* (Frietag *et al.*, 1985) and *N.gonorrhoeae* (Hagblom *et al.*, 1985). Coote and Brownlie (1988) suggested that serotype variation may assist the survival of the organism in hosts who lack immunity to the new variant. Foxall *et al.* (1990) suggested that DNA rearrangement by a DNA invertase could be involved in the mechanism of serotype conversion.



The *fim2* and *fim3* gene promoter regions are well conserved and contain a stretch of 13- 15 C-residues located just upstream of the putative -10 consensus region (Livey *et al.*, 1987; Mooi *et al.*, 1990). Analysis of this region has revealed that phase transitions between high and low levels of *fim* gene expression occur by insertions and deletions in the stretch of C-residues (Willems *et al.*, 1990). These authors propose that these mutations affect transcription of the *fim* genes by varying the distance between the binding site for an activator (possibly the *vir* gene product) and the -10 box.

#### Section 2.5. EXPRESSION IN *E.COLI* OF *B.PERTUSSIS* VIRULENCE DETERMINANTS.

A number of studies have shown that genes from a variety of bacterial pathogens can be transcribed and translated in *E.coli* e.g.

*Neisseria gonorrhoeae* (Meyer *et al.*, 1982)

*Vibrio cholerae* (Pearson and Mekalanos, 1982)

*Treponema pallidum* (Stamm *et al.*, 1982)

*Klebsiella pneumoniae* (Purcell and Clegg, 1983)

*Staphylococcus aureus* (Sako *et al.*, 1983)

*Bacillus anthracis* (Vodkin and Leppla, 1983)

*Legionella pneumophila* (Engleberg *et al.*, 1984).

Initial attempts to detect expression in *E.coli* of *B.pertussis* virulence determinants, without the use of expression vectors, were unsuccessful (Shareck and Cameron, 1984; Brownlie *et al.*, 1986; Loch and Keith, 1986; Nicosia *et al.*, 1986). These findings can be partly explained by the fact that *B.pertussis* virulence determinants require the *vir*-encoded

*trans*-activator for expression. Shareck and Cameron (1984) reported the cloning and expression in *E.coli* of two outer membrane proteins of 33kD and 30kD specific to *B.pertussis*. Brownlie *et al.* (1986) and Stibitz *et al.* (1988a) obtained expression in *E.coli* of several *B.pertussis* amino acid biosynthetic genes. These studies indicated that it was possible to obtain expression of some *B.pertussis* functions in *E.coli*.

Apart from the requirement for the *vir*-encoded *trans*-activator, there are several reasons which may explain why attempts to detect expression of PT in *E.coli* were unsuccessful (Locht and Keith, 1986; Nicosia *et al.*, 1986, 1987):

- i) the distance between the -35 and -10 consensus promoter sequences makes the *ptx* promoter inefficient in *E.coli*
- ii) the putative binding sites are not optimal for translation in *E.coli*
- iii) the codon usage does not resemble the codon usage of highly expressed genes in *E.coli*
- iv) some of the signal peptides of the PT subunits contain cysteine residues which may interfere with secretion and therefore cause a transcriptional arrest
- v) PT mRNA or subunit polypeptides may be unstable in *E.coli*.

Subunits of PT have been expressed in *E.coli* under the transcription and translation control of heterologous promoters (Barbieri *et al.*, 1987; Lochter *et al.*, 1987; Nicosia *et al.*, 1987; Burnette *et al.*, 1988). These studies indicated that the different codon usage of the *ptx* genes did not prevent their transcription and translation in *E.coli*. Nicosia and Rappuoli (1987) cloned the *ptx* promoter into a plasmid containing the *cat* gene, in order to determine its activity in *E.coli*. The wild-type *ptx* promoter was only weakly active in *E.coli*, however when a stretch of four C-residues was

deleted by site directed mutagenesis such that the *ptx* promoter contained optimal spacing of 17bp between the -35 and -10 regions, its activity was increased.

As well as PT subunit genes, other *B.pertussis* virulence genes which have been expressed in *E.coli* from heterologous promoters include *fha* (Reiser *et al.*, 1985; Mattei *et al.*, 1986), *cya* (Brownlie *et al.*, 1988; Glaser *et al.*, 1988a; Rogel *et al.*, 1989) and *fim2* (Walker *et al.*, 1990).

Stibitz *et al.* (1988a) demonstrated that expression in *E.coli* of the *B.pertussis fha* gene could be activated by *vir*. Miller *et al.* (1989) constructed an *E.coli* strain which contained a single copy transcriptional *fhaB::lacZYA* fusion chromosomally integrated on a recombinant lambda phage. Introduction of a multi-copy plasmid encoding *vir* resulted in a several hundredfold increase in  $\beta$ -galactosidase activity when compared with the vector control. However, expression of a transcriptional *ptxA::lacZYA* fusion in *E.coli* was not affected by the *vir* locus in-trans, which implied that the *B.pertussis ptx* and *fha* genes differed in their requirements for transcription. The *vir*-mediated activation of *fhaB::lacZYA* in *E.coli* was affected by the same environmental signals that modulate expression of virulence genes in *B.pertussis* (Miller *et al.*, 1989).

## Section 2.6. TWO-COMPONENT REGULATORY SYSTEMS.

Bacteria monitor continuously the composition of their environment, and adapt to changes. In many bacterial species, a diverse number of chemical and physical stimuli elicit a wide range of adaptive responses, such as changes in gene expression, cell morphology or cell movement. Genetic studies have indicated that such adaptive responses are controlled by members of two homologous families of proteins: a sensor protein (or histidine protein kinase) which acts as an environmental sensor that transmits a signal to its cognate regulator protein which effects the response (see Table 2.2). As described in Section 5.1.6, the *B.pertussis vir (bvg)* gene products share extensive homology with these two families of proteins (Arico *et al.*, 1989). The signal-transduction systems described here have been reviewed by Ronson *et al.* (1987), Stock (1987), Kofoed and Parkinson (1988), Bourret *et al.* (1989), Miller *et al.* (1989b) and Stock *et al.* (1989). Phosphorylation of the response regulator appears to be an essential feature of the signal transduction mechanism (see Bourret *et al.*, 1989; Stock *et al.*, 1989). Most of the regulator proteins act as positive regulators of transcription.

The sensor protein family share regions of conserved sequence of approximately 200 amino acids at their C-terminal end. Most members of the sensor class are transmembrane proteins with an N-terminal periplasmic domain which interacts with stimulatory ligands and transmembrane signals, and acts to control the kinase or phosphatase activities of its C-terminal conserved transmitter domain located in the cytoplasm. However, some sensor proteins are completely cytoplasmic in location e.g. NR11 (Ninfa *et al.*, 1986). The BvgC protein produced by *B.pertussis* (Arico *et al.*, 1989) and

Table 2.2

## Two-component regulatory systems.

System	Sensor	Regulator (class)	Adaptive response	Organism <sup>1</sup>	Reference(s) <sup>2</sup>
Ntr	NtrB (NRII)	NtrC (NRI)	nitrogen assimilation	Bp, Ec, Ka, Kp, St	Nixon et al. (1986) Ninfa and Magasanik (1986)
Omp	EnvZ	OmpR	porin gene expression	Ec, St	Comeau et al. (1985) Forst and Inouye (1988)
Vir	VirA	VirA/VirG	<i>Agrobacterium</i> virulence	At	Stachel and Nester (1986)
Pho	PhoR	PhoB	phosphate regulation	Ec	Makino et al. (1986a, b)
Fix	FixL	FixJ	nitrogen fixation	Rm	David et al. (1988)
Uhp	UhpB	UhpA	sugar phosphate transport	Ec	Weston and Kadner (1988)
Che	CheA	CheB/CheY	chemotaxis	Ea, Ec, St	Stock et al. (1985)
Spo	SpoIIJ	SpoOA/SpoOF	sporulation	Bs	Losick et al. (1986)

1. At, *Agrobacterium tumefaciens*; Bp, *Bradyrhizobium parasponiae*; Bs, *Bacillus subtilis*; Ea, *Enterobacter aerogenes*; Ec, *E. coli*; Kp, *Klebsiella pneumoniae*; Rm, *Rhizobium meliloti*; St, *Salmonella typhimurium*.
2. These systems are reviewed by Stock et al. (1989).  
Ka, *Klebsiella aerogenes*

sensor proteins such as VirA and CheA (see Stock *et al.*, 1989) have additional sequences beyond the C-terminal conserved transmitter domain.

The regulator proteins share a conserved receiver domain of approximately 120 amino acids extending from the N-terminus, which acts as the receptor of signals. Within the N-terminal domain of the CheY response regulator, a phosphoaccepting active site has been identified (see Stock *et al.*, 1989). The remainder of the regulator protein usually functions in DNA binding and interaction with RNA polymerase or its sigma factors. The family of regulator proteins can be subdivided into four classes, based on sequence similarities between their C-terminal domains (see Stock *et al.*, 1989):

Class I:- Members of this group e.g. NRI, have been shown to activate transcription from promoters that are recognized by  $E\sigma^{54}$  RNA polymerase holoenzyme. The NRI protein contains a region thought to be involved in DNA binding at its extreme C-terminus.

Class II:- Examples of this group include PhoB, OmpR and VirG. These proteins function to activate transcription of a specific set of target genes at promoters that are thought to be recognized by the major form of RNA polymerase, corresponding to  $E\sigma^{70}$  in *E.coli*. Like class I regulators, the C-terminal domains bind to specific DNA sequences upstream from the regulated promoters.

Class III:- This group includes UhpA and FixJ. These response regulators also function as transcriptional regulators, but their mechanisms of action are as yet unknown.

Class IV:- This group includes the CheY and SpoOF proteins which consist of only the conserved N-terminal domain. In these response regulators, a direct role for the activated response regulator domain is indicated.

Although signal transduction systems have been designated "two-component", some of these systems contain additional components e.g. the Uhp system of *E.coli* (Weston and Kadner, 1988) and the Bvg system of *B.pertussis* (Arico *et al.*, 1989) each contain three components. The ToxR system of *V.cholerae* is a mono-component analogue of the two-component system (Miller *et al.*, 1987; Taylor *et al.*, 1987). The *toxR* gene of *V.cholerae* coordinately controls cholera toxin, pilus and outer membrane expression. ToxR is a transmembrane protein, which has its transcriptional activation and DNA binding domains located in the N-terminal cytoplasmically located portion. The BvgC protein of *B.pertussis* also shows homology to both sensor and regulator proteins (Arico *et al.*, 1989).

Recently it has been demonstrated that supercoiling of cellular DNA varies in response to environmental stresses (Ni Bhriain *et al.*, 1989). These changes in DNA supercoiling appear to be responsible for regulating bacterial gene expression, and provide an underlying global regulatory network upon which more specific regulatory processes are superimposed. For example, regulation of expression of the *ompC* gene is mediated by two distinct mechanisms:- one involving the OmpR and EnvZ signal transduction system, and the other involving changes in DNA supercoiling (Ni Bhriain *et al.*, 1989). These two regulatory systems interact to determine the efficiency of productive initiation of transcription by RNA polymerase (Ni Bhriain *et al.*, 1989).

Section 2.7. OBJECT OF RESEARCH.

The main objectives of this research were:-

- i) To characterize clone pRMB2, and subclone the *vir* locus.
- ii) To determine whether the cloned *B.pertussis vir* locus *in-trans* would restore a Vir+ phenotype to avirulent phase variant strains of *B.pertussis* and *B.bronchiseptica*.
- iii) To determine the effect of multiple copies of *vir in-trans* in *B.pertussis* on the response to modulators.
- iv) To determine the ability of *vir* to *trans*-activate expression in *E.coli* of *B.pertussis* virulence genes.
- v) To analyse strains of *B.parapertussis*, *B.bronchiseptica* and *B.avium* for regions homologous to *vir*.



### Section 3: MATERIALS AND METHODS.

## Section 3.1. BACTERIOLOGICAL PROCEDURES.

### 3.1.1. Bacteria and plasmids.

The bacterial strains used in this study are listed in Tables 3.1 and 3.2; the plasmids used are listed in Table 3.3.

### 3.1.2. Growth of *Bordetella*.

For routine growth of *Bordetella* strains, Bordet-Gengou (BG) agar plates consisting of a layer of BG agar base (Gibco, BRL, Paisley, Scotland) overlaid with BG agar base containing 20% (v/v) defibrinated horse blood (Gibco, BRL) were used (see Appendix 1). Where appropriate, tetracycline (Tc) was added for plasmid selection and maintenance. *Bordetella* transconjugants were selected on BG agar containing Tc, cephalixin plus phage T1 suspension. BG agar containing kanamycin (Km) was used for selection of Tn5. To study the effect of nicotinic acid on modulation, BG agar containing nicotinic acid was used. *B.pertussis* strains were normally grown for 3- 5 days at 35°C. To determine the effect of low temperature on modulation, *B.pertussis* strains were grown for up to 7 days at 25°C. *B.bronchiseptica* strains were grown for 1- 2 days at 35°C. All cultures were incubated in a moist atmosphere.

Congo red medium (Parton, 1988, see Appendix 1) was used to determine the ability of several *B.pertussis* strains to uptake Congo red. Antibiotics were added as required. Plates were incubated in a moist atmosphere at 35°C for up to 7 days.

For preparation of genomic DNA, Stainer and Scholte medium (SS medium) was used. This medium was the '16G + 1P' medium originally described by Stainer and Scholte (1971). A loopful of growth from a BG agar culture was

Table 3.1  
***Bordetella* strains**

Strain	Characteristic(s)	Source or reference
<i>B. pertussis</i>		
TAB I	Virulent. Strain used for construction of the genomic library, from which clones pRMB2, pRMB1 and p26 were isolated. 1, 3 serotype.	Brownlie <i>et al.</i> (1985a)
BP347	Avirulent Tn5 mutant	Weiss <i>et al.</i> (1983)
Tohama I	Virulent. 1, 2 serotype. Ancestral strain of BP347.	Sato and Arai (1972)
L84 I	Virulent. 1, 2 serotype.	NCTC strain
L84 IV	Avirulent	NCTC strain
11615	Avirulent	Parton (1988)
44122/7R	Virulent	Branefors (1964)
44122/7R(34)	Avirulent, obtained by repeated passage (34 times) of strain 44122/7R.	J.G. Coote
BP353	FHA deficient Tn5 mutant	Weiss <i>et al.</i> (1983)
Wellcome 28	Virulent. 1, 2, 3 serotype	PHLS, CAMR

contd.

Table 3.1. (contd.)

Strain	Characteristic(s)	Source or reference
<i>B. paraptussis</i>		
59521	Virulent	NCTC strain
10520	Virulent	NCTC strain
<i>B. bronchiseptica</i>		
276 I	Virulent	Lax (1985)
FW5 I	Virulent	Lax (1985)
AS1 III	Avirulent	Lax (1985)
276 III	Avirulent	Lax (1985)
FW5 III	Avirulent	Lax (1985)
<i>B. avium</i>		
4091	Virulent	Rimler and Simmons (1983)
4148	Virulent	Rimler and Simmons (1983)
GOBL 118	Virulent	C. Gentry-Weeks
GOBL 124	Virulent	C. Gentry-Weeks
GOBL 136	Virulent	C. Gentry-Weeks
GOBL 141	Virulent	C. Gentry-Weeks
GOBL 142A	Virulent	C. Gentry-Weeks
GOBL 110	Avirulent, <i>B. avium</i> -like	C. Gentry-Weeks
GOBL 122	Avirulent, <i>B. avium</i> -like	C. Gentry-Weeks

Table 3.2

*E. coli* strains

Strain	Genotype	Source or reference
CAA8306	$\Delta$ <i>cya</i>	Kiely and O'Gara (1983)
DH1	<i>supE44 hsdR17 recA1 endA1</i> <i>gyrA96 thi-1 relA1</i>	Maniatis <i>et al.</i> (1982)
DH5	<i>supE44 hsdR17 recA1 endA1</i> <i>gyrA96 thi-1 relA1</i>	Hanahan (1985)
DH5 $\alpha$	a $\phi$ 80d <i>lacZ</i> $\Delta$ M15 derivative of DH5	Gibco, BRL
G806	$\Delta$ <i>cya</i>	Garges and Adhya (1985)
HB101	<i>supE44 hsdS20 recA13 ara14</i> <i>proA2 lacY1 galK2 rpsL20</i> <i>xyl-5 mtl-1</i>	Maniatis <i>et al.</i> (1982)
JA221	<i>hsdM<sup>+</sup> hsdR<sup>-</sup> lacY leuB6</i> <i>atrpe5 recA1</i>	Clarke and Carbon (1978)
JM83	<i>ara <math>\Delta</math>lac-pro strA thi</i> <i><math>\phi</math>80d<i>lacZ</i> <math>\Delta</math>M15</i>	Vieira and Messing (1982)

Table 3.3.

Plasmids

Plasmid	Characteristic(s)	Source or reference
pLAFR1	Broad-host-range cosmid cloning vector. 21.6kb. Tcr	Friedman <i>et al.</i> (1982)
pRK2013	<i>tra</i> functions of RK2 cloned in ColE1. Km <sup>r</sup>	Figurski and Helinski (1979)
pIC20H	Cloning vector containing a polylinker specifying 17 restriction sites in the $\beta$ -galactosidase $\alpha$ -complementing gene fragment. 2.7kb. Apr <sup>r</sup> .	Marsh <i>et al.</i> (1984)
pRK291	Broad-host-range cloning vector derived from RK2. Tcr.	Ditta <i>et al.</i> (1985)
pRK310	Broad-host-range cloning vector derived from RK2. Contains a polylinker sequence in the $\beta$ -galactosidase $\alpha$ -complementing gene fragment. Tcr.	Ditta <i>et al.</i> (1985)
pRMB2	Identified from a gene library of <i>B. pertussis</i> TAB7 DNA cloned in pLAFR1, by its ability to complement Vir <sup>-</sup> <i>B. pertussis</i> BP347.	Brownlie <i>et al.</i> (1988)
pDM3	A pLAFR1 derivative containing the 2.7kb <i>EcoRI</i> fragment (E3) of pRMB2.	this study

contd.

Table 3.3. (contd.).

Plasmid	Characteristic(s)	Source or reference
pDM30	A pIC20H derivative containing the 2.7kb <i>EcoRI</i> fragment (E3) of pRMB2.	this study
pDM1	A subclone constructed by digesting pRMB2 with <i>BamHI</i> , and ligating the largest fragment obtained (B6). Contains the <i>vir</i> locus.	this study
pDM62	A pIC20H derivative containing the 5.3kb <i>BamHI</i> to <i>EcoRI</i> fragment of pDM1.	this study
pDM623	A pIC20H derivative containing the 8.0kb <i>BamHI</i> to <i>EcoRI</i> fragment of pDM1.	this study
pDM14	A pIC20H derivative containing the 3.9kb <i>BamHI</i> to <i>EcoRI</i> fragment of pDM1.	this study
pBR328	Cloning vector. $\text{Apr}^r \text{Cm}^r \text{Tc}^r$	Soberon <i>et al.</i> (1980)
pIL22	A pBR328 derivative containing the <i>B. pertussis</i> serotype 2 fimbrial subunit gene.	Livey <i>et al.</i> (1987)
p26	A pLAFR1 derivative containing the <i>B. pertussis</i> AGG3 genetic determinant.	R. M. Brownlie
pRMB1	A pLAFR1 derivative containing the <i>B. pertussis</i> AC and HLY genetic determinants.	Brownlie <i>et al.</i> (1988)
contd.		

Table 3.3. (contd.)

Plasmid	Characteristic(s)	Source or reference
pRMB3	A pIC20H derivative containing the 10kb <i>Bam</i> HI fragment of pRMB1, cloned in the same orientation as the <i>lac</i> promoter.	Brownlie <i>et al.</i> (1988)
pRMB9	A pIC20H derivative containing the 3.0kb <i>Bam</i> HI to <i>Eco</i> RI fragment of pRMB1, cloned in the same orientation as the <i>lac</i> promoter.	Brownlie <i>et al.</i> (1988)
pRMB11	A pIC20H derivative containing the 6.0kb <i>Cla</i> I fragment of pRMB1, cloned in the opposite orientation to the <i>lac</i> promoter.	R.M. Brownlie
pRMB12	A pIC20H derivative containing the 6.0kb <i>Cla</i> I fragment of pRMB1, cloned in the same orientation as the <i>lac</i> promoter.	R.M. Brownlie
pUC19	Used as a control plasmid to determine transformation efficiencies. Apr.	Gibco, BRL



used to inoculate 50ml of SS medium contained in a 250ml dimpled flask. Flasks were shaken at 35°C for 48h in an orbital incubator.

Stocks of all *Bordetella* strains were stored at -70°C as cell suspensions in a 1ml solution of 1% casamino acids containing 15% glycerol (see Appendix 1). As required, frozen stocks were thawed and 100µl of cell suspension was spread on BG agar.

All strains were regularly checked for purity by Gram stain.

### 3.1.3. Growth of *E.coli*.

*E.coli* strains were routinely grown on nutrient agar (Oxoid) plates, or with shaking in nutrient broth (Oxoid). For selection and maintenance of plasmids, antibiotics were added as required. All *E.coli* strains were grown at 37°C.

For selection of *E.coli* JM83 or DH5α strains harbouring plasmids containing the *lac Z* (α) peptide coding region, nutrient agar containing the appropriate antibiotic, plus 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) was used. *E.coli* strains JM83 and DH5α synthesise an inactive C-terminal fragment of β-galactosidase, called an *omega* fragment. When such strains harbour *alpha* complementing vectors, the *alpha* and *omega* fragments form a complex that has β-galactosidase activity, and colonies appear blue on medium containing IPTG (which inactivates *lac* repressor and thus derepresses *omega* peptide synthesis) and X-gal, which is hydrolysed by β-galactosidase to the blue compound bromochloroindole. On nutrient agar containing X-gal and IPTG, recombinant clones can be easily identified as inactivation of the α peptide by cloned DNA yields a clearly discernible white colony.

For preparation of competent *E.coli* cells, strains were grown in SOC medium (Hanahan, 1983, see Appendix 1).

For storage of *E.coli* strains, 10ml of nutrient broth was inoculated with a single colony from a nutrient agar culture, and incubated overnight. The culture 0.5ml was added to an equal volume of sterile glycerol. The resulting suspension was mixed and stored at -20°C. As required, nutrient agar plates were inoculated with a loopful of cell suspension.

All strains were regularly checked for purity by Gram stain.

#### 3.1.4. Preparation of antibiotic solutions.

	<u>Stock solution</u>	<u>Working concentration</u>
Tetracycline (Tc)	10mg / ml	10µg / ml
Ampicillin (Ap)	25mg / ml	100µg / ml
Kanamycin (Km)	20mg / ml	20µg / ml
Cephalexin	5mg / ml	50µg / ml

Antibiotics were purchased from Sigma Chemical Co., Poole, Dorset, England.

Tc was dissolved in methanol. All other antibiotics used were dissolved in distilled water (d.H O). All antibiotic stock solutions were sterilised by membrane filtration<sup>2</sup> using a Minisart NML filter unit of pore size 0.2µM (Sartorius). Stock solutions were stored at -20°C.

#### 3.1.5. Preparation of nicotinic acid.

Nicotinic acid (Sigma) was prepared as a stock solution of 25mg/ml in d.H O, and sterilised by membrane filtration using a Minisart NML filter<sup>2</sup> unit, of pore size 0.2µM (Sartorius). Freshly prepared stock solution was diluted in BG medium to give a working concentration of 500µg/ml.

### 3.1.6. Preparation of X-gal and IPTG.

X-gal (Sigma) 100mg was dissolved in 4ml of N, N- dimethylformamide (Sigma) to give a stock solution of 25mg/ml. The stock solution was stored at -20°C. The working concentration of X-gal was 50µg/ml.

IPTG (Sigma) was prepared as a 0.5M stock solution in d.H O, sterilised by membrane filtration using a Minisart NML filter of pore size 0.2µM<sup>2</sup> (Sartorius), and stored at -20°C. The working concentration of IPTG was 1mM.

### 3.1.7. Phage T1 suspension.

Phage T1 suspension was obtained from Dr. J. G. Coote, and contained 5 x 10<sup>7</sup> plaque forming units of T1 phage per ml. For selection of *Bordetella* transconjugants, phage T1 was used as an added selection against *E.coli*. Just prior to use, 100µl of phage T1 suspension was spread on the surface of each BG agar plate, and allowed to dry at room temp for 6h.

## Section 3.2. GENETIC MANIPULATIONS.

### 3.2.1. Preparation of genomic DNA.

*B.pertussis* chromosomal DNA was prepared using a modification of the method of Hull *et al.* (1981), as described by Brownlie *et al.* (1986).

DNA from *B.pertussis* strains L84 IV, 11615, 44122/7R and 44122/7R(34), and from *B.parapertussis* strains was obtained from Dr. J. G. Coote. DNA from *B.bronchiseptica* strains was obtained from M. J. Ward. DNA from *B.avium* strains 4091 and 4148 was obtained from D. MacGregor. DNA from all other *B.avium* strains was kindly supplied by Dr. C. R. Gentry-Weeks, Washington University, St. Louis, Missouri, USA.

### 3.2.2. Large scale preparation of plasmid DNA.

The procedure outlined below is for a 500ml *E.coli* culture, but can be either scaled up or down. It is based on the alkaline lysis procedure of Birnboim and Doly (1979).

A single bacterial colony containing the plasmid of interest (or a loopful of -20°C stock) was used to inoculate 10ml of nutrient broth containing the appropriate antibiotic, and grown overnight at 37°C in an orbital shaker. Overnight culture 5ml was then transferred to 500ml nutrient broth containing the appropriate antibiotic, and incubated for 36h at 37°C in an orbital shaker. Bacterial cells were harvested by centrifugation at 9000 r.p.m. for 15 min at 4°C in a Sorvall GS-3 rotor. Cells were resuspended in 10ml of Solution I (50mM glucose, 25mM Tris-HCl (pH8.0), 10mM EDTA) containing 4mg/ml lysozyme (Sigma), and left at room temp for 5 min to allow bacterial lysis. Freshly prepared Solution II (0.2N NaOH, 1% (w/v) SDS) 20ml was added, mixed gently, and incubated on ice for 10 min. The solution was then neutralised by addition of 15ml of ice-cold Solution III (3M potassium acetate, pH5.0). The contents were mixed by shaking the closed centrifuge bottle several times, and incubated on ice for 10 min. The bacterial lysate was centrifuged at 8000 r.p.m. for 20 min in a Sorvall GS-3 rotor, to precipitate genomic DNA. The supernate was filtered through tissue into a 250ml centrifuge bottle, and 0.6 volumes of isopropanol were added and mixed well. The mixture was kept at room temp for 10 min. Nucleic acids were recovered by centrifugation at 8000 r.p.m. for 15 min at room temp in a Sorvall GSA rotor. The supernate was discarded, and the pellet was gently washed with 70% (v/v) ethanol. The pellet of nucleic acid was dissolved in 10ml TE buffer (10mM Tris-HCl (pH8.0), 1mM EDTA) and transferred to a 50ml centrifuge tube. Ten

millilitres of 1:1 (v/v) phenol:chloroform was added, and mixed to emulsion. (Phenol was purchased from Rathburn Chemicals Ltd., Walkerburn, Scotland, and equilibrated as described by Maniatis *et al.*, 1982.) The phases were separated by centrifugation at 5000 r.p.m. for 10 min in a Sorvall SS-34 rotor. The top, aqueous layer was removed and transferred to a clean tube. Ammonium acetate 5M 10ml was added and mixed thoroughly. The solution was incubated on ice for 15 min, then centrifuged at 10000 r.p.m. for 10 min at 4°C in a Sorvall SS-34 rotor. Isopropanol, 0.6 volume, was added to the supernate, mixed well, and incubated at room temp for 10 min. Nucleic acids were recovered by centrifugation at 10000 r.p.m. for 10 min at room temp in a Sorvall SS-34 rotor. The nucleic acid pellet was gently washed with 70% (v/v) ethanol, dried in a vacuum dessicator, redissolved in 0.5ml of TE buffer containing 50µg/ml RNase A, and incubated overnight at 37°C. (RNase A (Sigma) was prepared as a stock solution of 10mg/ml in 10mM Tris-HCl (pH7.5), 15mM NaCl, and heated to 100°C for 15 min to destroy DNase activity.) The mixture was extracted once with an equal volume of phenol, followed by one extraction with 1:1 (v/v) phenol:chloroform, and finally one extraction with chloroform alone. To precipitate the DNA, one tenth volume of 3M sodium acetate (pH5.2) and two volumes of ethanol were added (ethanol precipitation). The mixture was mixed by vortexing and incubated at -20°C for 2h. Precipitated plasmid DNA was recovered by centrifugation at 12000 r.p.m. for 15 min at room temp in a microfuge. The supernate was removed. Ethanol 70% (v/v) 0.5ml was added and mixed by vortexing. The mixture was centrifuged at 12000 r.p.m. in a microfuge. The plasmid DNA pellet was vacuum dried, and finally dissolved in 0.5ml TE buffer and stored at -20°C.

### 3.2.3. Small scale preparation of plasmid DNA.

The procedure outlined below is a modification of the method of Birnboim and Doly (1979). This method was used for plasmid analysis of *E.coli* transformant colonies.

Nutrient broth 10ml (containing the appropriate antibiotic(s)) was inoculated with a single *E.coli* colony, and incubated overnight at 37°C with shaking. Culture 1.5ml was transferred to a microfuge tube and cells were harvested by centrifugation at 12000 r.p.m. for 2 min in a microfuge. The cell pellet was resuspended in 100µl of lysis solution (50mM glucose, 25mM Tris-HCl (pH8.0), 10mM EDTA) containing 2mg/ml lysozyme. The suspension was mixed by vortexing, and kept at room temp for 2 min, to allow lysis. Freshly prepared Solution II (0.2N NaOH, 1% (w/v) SDS) 200µl was added. The contents were mixed by inverting the tube several times. The sample was incubated on ice for 5 min. Ice-cold Solution III (3M potassium acetate, pH5.0) 150µl was added, the contents of the tube were mixed by inversion and incubated on ice for a further 3 min. Precipitated genomic DNA and proteins were removed by centrifugation at 12000 r.p.m. for 5 min in a microfuge. Supernate 400µl was transferred to a clean microfuge tube containing 0.25ml isopropanol, to precipitate the DNA. Contents were mixed by vortexing. The sample was centrifuged at 12000 r.p.m. for 5 min. The supernate was removed and the DNA pellet was resuspended by vortexing in 0.5ml of 70% (v/v) ethanol. The sample was centrifuged at 12000 r.p.m. for 5 min. The supernate was removed and the pellet of double stranded DNA was dried in a vacuum dessicator. The nucleic acids were dissolved in 50µl of TE buffer containing 50µg/ml RNase A, and incubated at 70°C for 1h. This incubation helps inactivate bacterial DNases which may contaminate the plasmid DNA isolated in this way, and the RNase A digests contaminating

RNA. Diagnostic restriction endonuclease digestions were performed on 10 $\mu$ l aliquots of the crudely purified plasmid DNAs. The remainder of the plasmid preparation was stored at -20°C.

#### 3.2.4. Determination of nucleic acid concentrations.

The nucleic acid concentrations of genomic DNA preparations and of large scale plasmid preparations, were measured spectrophotometrically by determining the absorbance of an aqueous solution at 260nm and using the convention that an absorbance of 1 unit is equivalent to a double stranded DNA concentration of 50 $\mu$ g/ml (Maniatis *et al.*, 1982).

The nucleic acid concentrations of DNA fragments which had been purified from agarose gels were estimated by subjecting aliquots of an aqueous solution to electrophoresis, together with aliquots of lambda DNA (Gibco, BRL) of known concentration e.g. 100ng, 50ng and 10ng.

#### 3.2.5. Restriction endonuclease digestions.

*BscI* was purchased from NBL, Cramlington, Northumberland, England. All other restriction endonucleases were purchased from Gibco, BRL, Paisley, Scotland. Enzymes were used according to the manufacturers' instructions. For digestion of genomic DNA, 4mM spermidine (Sigma) was included in the reaction.

When only partial digestion by restriction endonucleases was required, a pilot digest was performed in a 100 $\mu$ l reaction volume containing 10 $\mu$ g of DNA. The reaction was pre-equilibrated at 37°C for 5 min prior to the addition of restriction enzyme (0.5 units/ $\mu$ g DNA). Samples (10 $\mu$ l) were removed from the digest at timed intervals after initiation of digestion, and immediately added to 10 $\mu$ l of 30mM EDTA (pH 8.0) on ice. To determine

the period of incubation which resulted in the greatest proportion of fragments in the desired size range, samples were analysed by electrophoresis.

### 3.2.6. Agarose gel electrophoresis of DNA.

Agarose gel electrophoresis of DNA for either analytical or preparative purposes was performed using flat bed apparatus (Pharmacia, LKB). Agarose (Type II-A: Medium EEO, Sigma) was dissolved in the appropriate buffer (by heating to 100°C) to give a final concentration of 0.7%. When preparative isolation of fragments was required, 1 x TAE buffer (0.04M Tris-acetate, 0.001M EDTA, pH8.0) was used. In all other instances, 0.5 x TBE buffer (0.045M Tris-borate, 0.001M EDTA, pH8.0) was used. The molten agarose was cooled to 50°C and poured into a horizontal gel mould (8cm x 10.5cm for 50ml of agarose, or 20cm x 20cm for 200ml of agarose), and allowed to solidify with an appropriate comb in place. Once solidified, the gel was submerged in the appropriate running buffer (1 x TAE or 0.5 x TBE) and DNA solution containing 1/10th volume of 10 x gel loading buffer (60% (w/v) sucrose /0.1% (w/v) bromophenol blue, in H<sub>2</sub>O) loaded into the wells. To give molecular weight standards, a sample of *HindIII*- or *PstI*- digested  $\lambda$  DNA was also loaded. (For molecular sizes of  $\lambda$  DNA restriction fragments, see Appendix 2.) Gels of 50ml volume were usually run at 100V for 2h, and gels of 200ml volume were usually run at 40V overnight. Following electrophoresis, gels were soaked in ethidium bromide solution (0.5µg/ml) for 15- 30 min, and excess ethidium bromide removed by soaking the gel in d.H<sub>2</sub>O for 10 min. DNA was then visualised by illumination with short wave ultra violet (u.v.) light, and photographed through a red filter using Polaroid type 667 or 665 film.



When fragment isolation from low melting-point agarose gels was required, agarose (Type I: Low EEO, Sigma) was used. These gels were prepared in a similar fashion, but were poured and subjected to electrophoresis at 4°C.

### 3.2.7. Estimation of molecular size of DNA fragments.

To estimate the length of DNA fragments, a relationship has to be established between DNA length and electrophoretic mobility in agarose gels, for standard fragments. This relationship is then used to calculate the molecular size of unknown fragments from their mobilities.

For construction of restriction enzyme maps of clones pRMB2 and pDM1 (Section 4.2.2), a modification of the method of Plikaytis *et al.* (1986), (D. J. Platt, personal communication) was used for accurately estimating the molecular sizes of restriction fragments after electrophoresis. Band migration distances were measured from a photograph of the ethidium bromide stained gel using a soft laser scanning densitometer. *HindIII*-digested  $\lambda$  DNA, or the 11.5kb to 0.15kb fragments of *PstI*-digested  $\lambda$  DNA were used as molecular weight standards. Data was fitted to a robust modified hyperbola based on the algorithm of Schaffer and Sederoff (1981). The modified BASIC program and use of an IBM PC were kindly provided by Dr. D. J. Platt, Dept. of Bacteriology, Glasgow Royal Infirmary, Glasgow.

A second method was used in some instances e.g. Section 4.3.1. The molecular sizes of restriction fragments were estimated from a graph of migration distance versus log DNA length, plotted for *HindIII*-digested  $\lambda$  DNA molecular weight standards.

### 3.2.8. Isolation of DNA fragments from agarose gels.

Samples of DNA were mixed with 1/10th volume of 10 x gel loading buffer, and subjected to agarose gel electrophoresis using 1 x TAE buffer. After electrophoresis, gels were stained with ethidium bromide, and DNA was visualised in u.v. light. A slice of agarose containing the band of interest was cut out using a sharp scalpel. Three different methods were used to purify DNA fragments from agarose gel slices. The recovery of DNA was checked in each instance by subjecting an aliquot of the purified DNA fragments to agarose gel electrophoresis.

#### 1) Recovery of DNA from low melting-point agarose.

Purification of DNA from low melting-point agarose was carried out essentially as described by Maniatis *et al.* (1982). The gel slice containing the band of interest was placed in a microfuge tube. Approximately 5 volumes of buffer (20mM Tris-HCl (pH8.0), 1mM EDTA) were added and the sample incubated at 65°C for 5 min to melt the gel. The solution was cooled to room temp, then extracted once with phenol, followed by once with 1:1 (v/v) phenol:chloroform, and finally once with chloroform alone. To precipitate the DNA, one tenth volume of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol were added to the aqueous phase, mixed by vortexing, and incubated at -20°C for 2h. DNA was then recovered by centrifugation at 12000 r.p.m. for 15 min in a microfuge. The DNA pellet was washed once with 70% (v/v) ethanol, vacuum dried, and redissolved in an appropriate volume of d.H O. This method was routinely used to prepare DNA fragments for restriction endonuclease analysis, or for cloning.

## 2) Recovery of DNA by centrifugation through cellulose acetate.

The agarose gel slice containing the band of interest was placed in a SPIN-X centrifuge filter unit (cellulose acetate membrane 0.45µm, Costar, Cambridge, Massachusetts) and centrifuged at 12000 r.p.m. for 15 min at room temp in a microfuge. The aqueous DNA solution was recovered and transferred to a clean microfuge tube. DNA was recovered by ethanol precipitation, and finally redissolved in an appropriate volume of TE buffer. This method was developed by Dr. A. Jenkins, and was used to prepare DNA fragments for oligo-labelling (Section 3.2.10).

## 3) Recovery of DNA by electroelution.

Isolation of DNA fragments from agarose using the SS-BIOTRAP apparatus (Schleicher & Schuell) was tried as an alternative to fragment isolation from low melting-point agarose. The SS-BIOTRAP apparatus was assembled and used as recommended by the manufacturer. The agarose gel slice containing the band of interest was placed in the elution chamber. A voltage of 150 V was applied to the electrophoresis chamber for 3h, to elute the DNA from the gel slice. The eluate was recovered and transferred to a microfuge tube. DNA fragments were recovered by ethanol precipitation, and finally redissolved in an appropriate volume of d.H O. Fragments isolated by this method were successfully used for cloning (see Section 4.7.3).

### 3.2.9. Southern blotting and hybridisation.

To give molecular weight standards,  $\lambda$  DNA was digested with *HindIII* or *PstI*, and labelled with <sup>32</sup>P-dATP, essentially as described by Downing et al. (1979). Briefly, a reaction containing 7µl of digested  $\lambda$  DNA (approximately 10ng), 26µl of d.H O, 10µl of  $\bar{A}$  buffer (mixture containing

0.1mM each of dCTP, dGTP, dTTP), 5µl REact 2 buffer (50mM Tris-HCl pH8.0, 10mM MgCl<sub>2</sub>, 50mM NaCl, Gibco, BRL), 1µl α-<sup>32</sup>P-dATP (10µCi/µl, Amersham) and 1µl Klenow fragment of DNA polymerase I (Boehringer, Mannheim) was incubated at room temp for 30 min.

A 10µl aliquot of <sup>32</sup>P-dATP-labelled *HindIII*- (or *PstI*-) digested λ DNA was loaded on a gel, together with restriction endonuclease digested genomic or plasmid DNA samples. (All samples contained a 1/10th volume of gel loading buffer.) Samples were subjected to electrophoresis through 0.7% agarose, at 40V overnight in 0.5 x TBE buffer. After staining in 0.5µg/ml ethidium bromide, the gel was photographed, and vacuum blotted using the VacuGene Vacuum Blotting System (LKB 2016, Pharmacia LKB) exactly as recommended by the manufacturer. Nitrocellulose membrane (Schleicher & Schuell) was routinely used for Southern blotting. However, as an alternative, Hybond-N blotting membrane (Amersham) was used for Southern blot analysis of *B. avium* DNA (Section 4.3.2). Successful transfer was confirmed by restaining the electroblotted gel in ethidium bromide solution. After transfer, DNA was fixed to the nitrocellulose membrane by baking in a vacuum oven at 80°C for 2h. The Hybond-N membrane was wrapped in Saran wrap (Dow Chemical Company) and placed DNA-side down on a u.v. transilluminator for 2 - 5 min to fix the DNA.

Each filter was sealed in a bag with 12ml of prehybridisation buffer [2.5ml of 20 x SSC (3M NaCl, 0.3M Na citrate, pH7.0), 6.0ml of formamide (purchased from Gibco, BRL, and deionised as described by Maniatis *et al.*, 1982), 1.2ml of 50 x Denhardt's reagent (1% (w/v) Ficoll (Sigma), 1% (w/v) polyvinylpyrrolidone (Sigma), 1% (w/v) BSA (Gibco, BRL)), 0.25ml of 20% (w/v) SDS, 0.2ml of 10mg/ml denatured salmon sperm DNA (prepared as described by Maniatis *et al.*, 1982) and d.H<sub>2</sub>O to 12ml ] and submerged in a

water bath at 42°C overnight. Following incubation, as much prehybridisation buffer as possible was squeezed out of the bag. Oligo-labelled DNA 50µl was added to 12ml of hybridisation buffer (2.5ml of 20 x SSC, 6.0ml of deionised formamide, 1.2ml of 50 x Denhardt's solution, 0.25ml of 20% (w/v) SDS, 0.25ml of 0.5M EDTA (pH8.0) and d.H O to 12ml). This mixture was then added to the filter. The bag was re-sealed and submerged in a water bath at 42°C for a further 24h.

The filter was removed from the bag and immediately submerged in 200ml of 2 x SSC, 0.1% (w/v) SDS and incubated at room temp for 30 min with gentle agitation. The filter was then washed in 200ml of 1 x SSC, 0.1% (w/v) SDS in a water bath at 68°C for 2h with gentle agitation. Filters were wrapped in Saran wrap and exposed to X-ray film (X-OMAT-S, Kodak) at -70°C overnight, to obtain an autoradiographic image. The use of a Kodak X-OMAT film processor to develop autoradiographs was kindly provided by The Beatson Institute for Cancer Research, Bearsden, Glasgow.

Where it was necessary to rehybridise filters, residual radioactivity was removed by immersing the nitrocellulose membrane in 30mM NaOH for 5 min, then briefly in 0.5M Tris-HCl (pH7.5), 1.5M NaCl, followed by immersion in 0.1 x SSC, 0.1% (w/v) SDS for 5 min. Residual radioactivity was removed from Hybond-N nylon membrane (Amersham) following the manufacturer's instructions. Filters were exposed to autoradiography to confirm that residual radioactivity had been removed, then prehybridised as before.

### 3.2.10. Oligo-labelling of DNA.

A technique has been developed (Feinberg and Vogelstein, 1983 and 1984) for radiolabelling DNA restriction endonuclease fragments to high specific

activity. Plasmid DNA (purified as described in Section 3.2.2) or DNA restriction fragments (purified by centrifugation through a SPIN-X filter unit) were radiolabelled as described by Feinberg and Vogelstein (1984). DNA 40ng was brought to a volume of 34 $\mu$ l with d.H O, denatured by boiling in H O for 5 min and quenched on ice. Oligo-labelling buffer (obtained from M. Ward and prepared as described by Feinberg and Vogelstein, 1984) 10 $\mu$ l, 2 $\mu$ l of 10mg/ml BSA (Gibco, BRL), 2 $\mu$ l of  $\alpha$ -<sup>32</sup>P-dATP (10 $\mu$ Ci/ $\mu$ l, Amersham) and 2 $\mu$ l of Klenow fragment of DNA polymerase I (Boehringer, Mannheim) were added. The reaction was mixed well, and incubated at room temp for 5h. The reaction was boiled in H O for 5 min, quenched on ice, and immediately used for Southern blot hybridisation.

### 3.2.11. Dephosphorylation of plasmid DNA.

In order to prevent vectors from self-ligating during cloning, the 5' phosphate groups of linear double-stranded DNA were removed by treatment with calf intestinal alkaline phosphatase (CIP, Boehringer, Mannheim).

Plasmid DNA 10 $\mu$ g was digested to completion with the appropriate restriction endonuclease(s). For vectors cut with only one enzyme, the digestion mixture was extracted once with phenol, once with 1:1 (v/v) phenol:chloroform, followed by once with chloroform alone. The aqueous phase was ethanol precipitated, and the recovered DNA finally redissolved in 180 $\mu$ l of d.H O. For vectors cut with two different restriction enzymes, the digestion mixture was subjected to agarose gel electrophoresis. Vector DNA was purified from the gel using the SS-BIOTRAP apparatus, and finally redissolved in 180 $\mu$ l d.H O.

Twenty microlitres of 10 x phosphatase buffer (100mM Tris-HCl (pH9.2), 1mM EDTA) was added to the 180 $\mu$ l of digested plasmid DNA. This mixture was

divided in two. To one aliquot, 1 $\mu$ l (10 units) of CIP was added. To the second aliquot, 1 $\mu$ l of d.H O was added (to give a non CIP-treated control). The mixtures were incubated at 37°C for 30 min, then 70°C for 60 min (to inactivate the enzyme). Samples were ethanol precipitated. Recovered vector DNA samples were redissolved at the desired concentration in d.H O. To check DNA recovery, a small aliquot from each sample was analysed by agarose gel electrophoresis.

On occasion, as an alternative to the above procedure, vector DNA was dephosphorylated according to the protocol recommended by the manufacturers of CIP (Boehringer, Mannheim).

To test whether vector DNA had been dephosphorylated to a satisfactory extent, samples of ligated CIP-treated and non CIP-treated vector DNA were transformed into competent *E.coli* cells.

### 3.2.12. Ligation of DNA.

Ligations were performed at room temp for either 2h (for ligation of overhanging ends) or overnight (blunt end ligations) in 20mM Tris-HCl (pH7.6), 10mM MgCl<sub>2</sub>, 10mM DTT, 1mM ATP, 5% PEG 8000, using 1 unit of T4 DNA ligase (Boehringer, Mannheim).

Ligation reactions were done in volumes of 10 $\mu$ l. For ligation of DNA fragments into vectors, reactions contained a molar excess of insert DNA. (One kilobase pair of DNA is  $6.49 \times 10^5$  daltons.)

### 3.2.13. Preparation of competent cells.

Transformation-competent *E.coli* cells were prepared using a modification of the method of Mandel and Higa (1970).

A single bacterial colony was used to inoculate 10ml of SOC medium

(Hanahan, 1983, see Appendix 1) and grown overnight at 37°C with shaking. The culture was diluted 1 in 100 in 50ml of SOC medium, and incubated as above. The culture was grown to an OD<sub>550</sub> of 0.2 or 0.5, for *rec+* or *rec-* strains respectively. The culture was chilled on ice for 10 min, then bacterial cells were harvested by centrifugation at 5000 r.p.m. for 5 min at 4°C in a Sorvall SS-34 rotor, resuspended in 25ml of chilled, sterile 50mM CaCl<sub>2</sub>, incubated on ice for 15 min, then pelleted again and resuspended in one tenth of the original culture volume of chilled, sterile 50mM CaCl<sub>2</sub>. Transformation-competent cells were stored at 4°C for 2h before use. Each batch of competent cells was usually transformed with 1ng and 10ng amounts of plasmid pUC19 DNA (Gibco, BRL) to allow the transformation efficiency (expressed as the number of transformants obtained per µg of DNA) to be determined.

#### 3.2.14. Transformation of competent cells.

The 10µl ligation reaction was placed on ice. Ice-cold TE buffer 40µl, 20µl of ice-cold TCM buffer (100mM Tris-HCl (pH7.0), 100mM MgCl<sub>2</sub>, 100mM CaCl<sub>2</sub>) and 130µl of freshly prepared competent *E.coli* cells were added and mixed gently. This suspension was incubated on ice for 20 min, heat-shocked at 42°C for 1.5 min, then placed on ice for 2 min. One ml of SOC medium was added, and the mixture shaken at 37°C for 60 min to allow expression of the plasmid-encoded antibiotic resistance. The mixture was spun briefly in a microfuge (30 sec) and the cells resuspended in 100µl SOC medium and plated onto nutrient agar plates containing the appropriate antibiotic (plus X-gal and IPTG if required).

For transformation of only a 1µl aliquot from a ligation reaction (or a 1µl aliquot of supercoiled plasmid DNA), 20µl of competent *E.coli* cells was



added directly to the chilled 1µl sample. The sample was incubated on ice for 20 min, 42°C for 1.5 min, and on ice for 2 min. SOC medium 80µl was added, incubated at 37°C for 1h, and the whole sample was plated out on selective nutrient agar.

As a negative control, the appropriate selective nutrient agar plate was inoculated with competent *E.coli* cells. All plates were incubated at 37°C overnight.

### 3.2.15. Conjugation of *E.coli* and *Bordetella*.

Broad host-range vectors pLAFR1 and pRK310 are not self-transmissible, therefore a tri-parental mating system was used which included a helper plasmid pRK2013, which contains the RK2 *tra* genes cloned in ColE1 (Figurski and Helinski, 1979).

Equal portions of fresh overnight cultures of the appropriate *E.coli* donor strain, and helper strain *E.coli* HB101 (pRK2013) were mixed and diluted 1 in 10 in sterile saline. A BG agar plate supporting a 48h lawn culture of the appropriate *B.pertussis* recipient strain (or a 24h lawn culture of the appropriate *B.bronchiseptica* recipient strain) was gently flooded with approximately 3ml of the *E.coli* donor and helper strain mixture. The excess was removed, and the plate incubated at 35°C for either 8h or overnight. A loopful of cells was transferred to 1ml of sterile saline, and 100µl of the resulting suspension was plated out on selective BG agar containing Tc, cephalixin and phage T1.

Where it was necessary to transfer a large number of clones by conjugation from *E.coli* to *B.pertussis* (e.g. Section 4.1.6), a microtitre tray system was used. A multi-point inoculator was used to inoculate fresh BG agar plates from lawn cultures of the *B.pertussis* recipient strain.

Plates were incubated at 35°C for 48h. *E.coli* donor strains were inoculated into the wells of a Cooke microtitre tray containing 50µl per well of nutrient broth + Tc, and incubated at 37°C overnight. Fresh overnight culture of *E.coli* HB101 (pRK2013) 50µl was added to each well. Using the multi-point inoculator, the *E.coli* donor and helper strain mixtures were added to the BG agar plates supporting "spots" of growth of the *B.pertussis* recipient strain, and incubated at 35°C overnight. Using the multi-point inoculator, cells were transferred to selective BG agar.

As controls, selective BG agar plates were inoculated with donor, helper and recipient strains. All plates were incubated at 35°C, in a moist atmosphere, for 2 - 5 days.

#### 3.2.16. Conjugation of *E.coli* and *E.coli*.

A tri-parental mating system, which utilised the *tra* functions of helper plasmid pRK2013, was used to transfer clone pRMB2 from donor strain *E.coli* DH1 (pRMB2) to recipient strain *E.coli* JA221 (pIL22) (Section 4.7.1).

Aliquots 50µl from fresh overnight cultures of *E.coli* donor, helper and recipient strains were mixed and spread on the surface of a nutrient agar plate. The plate was incubated, surface uppermost, at 37°C for 5h. A loopful of cells was transferred to 1ml of sterile saline, and 100µl of the resulting suspension was plated out on selective nutrient agar containing Ap plus Tc. As negative controls, donor, helper and recipient strains were plated out on selective nutrient agar. All plates were incubated at 37°C overnight.

### Section 3.3. MISCELLANEOUS MATERIALS AND METHODS.

#### 3.3.1. Treatment of samples for SDS-PAGE.

For *B.pertussis* samples, cells were harvested from a 48h BG agar culture into 1ml of physiological saline. For *E.coli* samples, cells were harvested by centrifugation from a 50ml culture (in the exponential phase of growth), and resuspended in 1ml of saline. Cells were washed once with saline and resuspended to give an OD<sub>660</sub> of 5.0 (about 2mg protein/ml). Samples were mixed with an equal volume of solubilising buffer (125mM Tris-HCl (pH6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v)  $\beta$ -mercaptoethanol, 0.002% (w/v) bromophenol blue) and heated to 100°C for 5 min prior to loading on the gel.

#### 3.3.2. SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis).

The method used was based on that of Laemmli (1970), using a discontinuous buffer system. Stacking and separating gels were prepared using a stock solution of 30% (w/v) acrylamide, 0.8% (w/v) N,N'-bis-methylene acrylamide, and contained 5% (w/v) and 11% (w/v) acrylamide respectively. The gel was formed between two glass plates of 17cm x 19cm x 0.3cm. Gels and electrophoresis buffer contained 0.1% (w/v) SDS.

Samples (30 $\mu$ l) were loaded onto the gel. To estimate molecular weights of separated polypeptides, a sample of molecular weight markers (SDS-6, Sigma) was loaded on one track of the gel. This contained a mixture of bovine serum albumin (66kD), egg albumin (45kD), pepsin (34.7kD), trypsinogen (24kD),  $\beta$ -lactoglobulin (18.4kD) and lysozyme (14.3kD). Electrophoresis was performed at room temp at a constant current of 30mA

until the dye front reached the bottom of the gel.

After electrophoresis, gels were stained overnight at room temp with Coomassie Blue stain (0.25% (w/v) Coomassie Blue R250 (BDH), 50% (v/v) methanol, 10% (v/v) glacial acetic acid), then destained using several changes of destaining solution (5% (v/v) methanol, 7.5% (v/v) glacial acetic acid).

Where subsequent analysis of samples by immunoblotting was required, duplicate samples were loaded on each half of the gel. Following electrophoresis, one half of the gel was treated with Coomassie Blue stain, and the other half was used for Western blotting.

### 3.3.3. Western blotting and immunological detection of proteins.

Western blotting was performed by the method of Towbin *et al.* (1979) using a "Transblot" transfer apparatus (Bio-rad). A sheet of nitrocellulose (Schleicher & Scheull) and two sheets of filter paper (3MM, Whatman) were cut to the same size as the SDS-polyacrylamide gel, and soaked in pre-cooled transfer buffer (25mM Tris, 0.2M glycine, 20% (v/v) methanol, pH8.3). The gel and the nitrocellulose membrane were "sandwiched" together between the two sheets of filter paper, and assembled in the "transblot" cassette. The cassette was then placed in the electrophoresis chamber containing pre-cooled transfer buffer, with the nitrocellulose facing the anode. Proteins were transferred at a power setting of 80mA overnight at room temp with a cooling system operating within the electrophoresis chamber. Successful transfer was confirmed by the absence of bands after staining the electroblotted gel in Coomassie Blue stain.

The nitrocellulose was immediately immersed in TTS buffer (20mM Tris, 500mM NaCl, 1% (v/v) Tween-20 (Sigma), pH7.2) and incubated overnight at

4°C. The filter was transferred to TBS buffer (20mM Tris, 500mM NaCl, pH7.2) containing 2% (v/v) inactivated horse serum (Sigma), and incubated at room temp for 1h with gentle agitation, then incubated for 90 min at room temp with a 1/100 dilution (in TBS buffer with 2% (v/v) inactivated horse serum) of primary antibody i.e. "Preston 2" or "Preston 3" polyclonal antisera (obtained from Dr. N. Preston) which were raised in rabbits and absorbed until monospecific for *B.pertussis* AGGs 2 and 3 respectively. The filter was washed in TBS buffer with 5 changes over a 30 min period, incubated with a 1/500 dilution (in TBS buffer with 2% (v/v) inactivated horse serum) of <sup>goat</sup> anti-rabbit IgG horse-radish peroxidase enzyme conjugate (HRP, Scottish Antibody Production Unit, Carlisle, UK) for 90 min, then washed in TBS buffer with 5 changes over 30 min. The filter was then incubated with substrate (one part of 3mg/ml HRP colour development reagent, 4-chloro-1-naphthol (Sigma), in methanol mixed with five parts 0.018% H<sub>2</sub>O<sub>2</sub> in TBS immediately before use) for 30 min. The nitrocellulose filter was finally washed with d.H<sub>2</sub>O, air dried and stored.

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#### 3.3.4. ELISA (enzyme-linked immunosorbent assay).

Fresh overnight cultures of *E.coli* strains were diluted 1 in 100 in 50ml nutrient broth (containing the appropriate antibiotic(s)) and incubated at 37°C with shaking until the exponential phase of growth was reached. Cells were harvested by centrifugation, washed once in saline, and resuspended to give an OD<sub>660</sub> of 5.0. Cells were harvested from 48h BG agar cultures of *B.pertussis* into 1ml of saline, washed and resuspended as above.

Samples of washed cells 200µl were applied, in duplicate, to separate wells of an assembled Bio-Dot apparatus (Bio-rad), and gentle suction was

applied to filter the cells onto the nitrocellulose membrane (Schleicher & Schuell).

Filters were placed above a dish of chloroform for 10 min to lyse the cells, and incubated at 4°C overnight in PBS (8.0 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na HPO<sub>2</sub>, 0.2 g/l KH PO<sub>2</sub>) containing 5% (w/v) BSA. Filters were washed at room temp in PBS, with 3 changes over a 45 min period, then incubated for 2h with a 1/10 dilution (in PBS with 1% (w/v) BSA) of primary antibody i.e. monoclonal antibody Ag2A (which was raised in mice against purified inactive serotype 2 fimbriae, and kindly supplied by PHLS, CAMR) or polyclonal "Preston 2" antibody. Filters were washed in PBS as before then incubated for a further 2h with a 1/2000 dilution (in PBS with 1% (w/v) BSA) of anti-mouse (for Ag2A antibody) or anti-rabbit (for "Preston 2" antibody) IgG horse-radish peroxidase enzyme conjugate (Scottish Antibody Production Unit). Filters were again washed in PBS, then incubated with substrate (2 mg/ml 3- amino- 9- ethylcarbazole (Sigma), 0.024% (v/v) H<sub>2</sub>O in 50mM sodium acetate, pH 5.0) for 30 min. The reaction was terminated after 30 min by placing the filters in d.H<sub>2</sub>O. Filters were air dried and stored.

### 3.3.5. Assay for FHA.

FHA activity was assayed by the ability of bacterial cell samples to agglutinate washed horse erythrocytes. Cells were harvested from either a 48h BG agar culture (for *Bordetella*) or from a nutrient broth culture in the exponential phase of growth (for *E.coli*), washed once with PBS, and resuspended in PBS to give an OD<sub>660</sub> of 5.0 i.e. approximately 2mg protein per ml. A 2ml sample of defibrinated horse blood (Gibco, BRL) was washed three times with PBS, the erythrocyte pellet being finally resuspended to

give a 2% (v/v) suspension.

A series of two-fold dilutions for each test sample was set up in the wells of a round-bottomed Cooke microtitre tray. An equal volume (50µl) of erythrocyte suspension was added to each well and mixed. As negative controls, wells were set up which contained erythrocyte suspension plus PBS only. Haemagglutination was assessed after incubation at room temp for 1h.

#### 3.3.6. Slide agglutination assay.

A slide agglutination assay was used to determine agglutinin 1 (AGG 1) and agglutinin 2 (AGG 2) activity. Bacterial cells were harvested and resuspended in saline as described in Section 3.3.4. To act as a negative control, a drop of saline was placed at one end of a clean microscope slide. At the other end, a drop of antisera was placed i.e. "Preston 1" or "Preston 2" antisera (obtained from Dr. N. Preston) which were raised in rabbits and absorbed until monospecific for *B.pertussis* AGGs 1 and 2 respectively. A drop of bacterial cell suspension was added to each, the slide was rocked gently, and agglutination assessed by comparing the test reaction with the negative control.

#### 3.3.7. Assay for HLY.

Haemolysin activity was visualised after growth on BG agar.

#### 3.3.8. Assay for HLT.

*B.pertussis* cells were harvested from 48h BG agar cultures into PBS, to a final concentration of 10 opacity units (OU) by comparison with the WHO 5th International Reference Preparation of Opacity (Perkins et al. 1973). Assay for HLT was then kindly performed by Dr. R. Parton, as described by Parton (1988).

### 3.3.9. Assay for PT.

*B.pertussis* suspensions were prepared to a concentration of 10 OU as described above, then heated to 56°C for 30 min to destroy heat-labile toxin. Assay for PT was then kindly performed by Dr. R. Parton, as described by Parton (1988).

### 3.3.10. Assay for X-OMPs.

To determine the presence of the X-mode specific envelope polypeptides of molecular weights 28 kD and 30 kD, whole cell *B.pertussis* samples were subjected to SDS-PAGE. Polypeptides were visualised by Coomassie Blue staining.

### 3.3.11. Assays for AC enzymic activity.

#### Microtitre tray assay for AC production.

AC production was assayed by the development of a blue colour from X-gal in the wells of microtitre trays. This relies on expression of  $\beta$ -galactosidase in *E.coli* G802 ( $\Delta cya$ ) which is dependent on cAMP produced by exogenous AC (C. Duggleby, personal communication).

*Bordetella* cultures were harvested from BG agar, washed once in saline, and resuspended to an OD of 5.0. Duplicate 8 $\mu$ l aliquots for each *Bordetella* test sample were added to 50 $\mu$ l aliquots of a reaction mixture (60mM Tris-HCl pH 8.0, 10mM MgCl<sub>2</sub>, 5mM ATP, 10mM CaCl<sub>2</sub>, 500 units per ml calmodulin (Sigma)) contained in the wells of a microtitre tray, mixed, and incubated at 35°C for 3h. A test culture was prepared by inoculating fresh nutrient broth containing IPTG (250 $\mu$ g/ml) and X-gal (250 $\mu$ g/ml) with a 1 in 100 dilution of a 6h culture of *E.coli* G802. Test culture 150 $\mu$ l was added to each well, and the tray was incubated overnight at 35°C. Dark blue cultures were recorded as positive.



Assay for AC production using a cAMP assay kit.

The enzymic activity of *B.pertussis* AC in *E.coli* (Section 4.7.5) was measured in an assay which involved two steps: incubation of the sample with ATP, the substrate from which the adenylate cyclase generates cAMP, and then measurement of the cAMP generated by a competitive binding assay involving radiolabelled cAMP.

*E.coli* cells were harvested from late exponential phase cultures, resuspended in 8M urea to a final concentration of 0.16g/ml, and sonicated three times for 30 sec using a Branson sonifier at maximal output. The protein concentration of each sample was determined by the method of Bradford (1976). Samples were diluted 200-fold in PBS.

To convert ATP to cAMP, a modification of the method described by Brownlie *et al.* (1985a) was used. A reaction containing 120 $\mu$ l of 0.002M ATP in 0.02M MgCl<sub>2</sub>, 80 $\mu$ l of 0.1M tricine (pH8.0), 40 $\mu$ l *E.coli* extract and 250 units of calmodulin (Sigma), was set up for each *E.coli* sample. Reactions were mixed, incubated at 30°C for 15 min, then terminated by adding 480 $\mu$ l of 50mM Tris (pH7.5), 0.5M EDTA, and heating at 100°C for 5 min.

The cAMP content of samples, generated from the above reaction, was then assayed using a cAMP assay kit (Amersham). This assay is based on competition between unlabelled cAMP and a fixed quantity of tritium labelled cAMP for binding to a protein which has a high specificity and affinity for cAMP. The amount of labelled protein-cAMP complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. Measurement of the protein bound radioactivity enables the amount of unlabelled cAMP in the sample to be determined from a linear standard curve.

#### Section 4: RESULTS.

## Section 4.1. MOLECULAR ANALYSIS OF pRMB2 AND SUBCLONING OF THE VIR LOCUS.

### 4.1.1. Identification of the site of Tn5 insertion in BP347.

Clone pRMB2 was identified from a gene library of *B.pertussis* TAB I DNA by its ability to complement the Vir- mutation of Tn5 mutant BP347 (Brownlie *et al.*, 1988). Hybridisation analysis was used to determine the region of clone pRMB2 showing homology to the site of Tn5 insertion in BP347, and thereby define the region essential to the *vir* locus. The gene library (in *E.coli* DH1) was constructed by cloning genomic DNA which had been partially digested with *EcoRI*, into the unique *EcoRI* site of cosmid vector pLAFR1 (Brownlie *et al.*, 1986). As Tn5 contains no *EcoRI* sites, this enzyme was chosen for preliminary analysis of pRMB2 and chromosomal DNA.

Plasmid DNA was prepared from *E.coli* DH1 (pRMB2), and genomic DNA was prepared from *B.pertussis* strains L84 I, BP347, Tohama I (the parent strain of BP347) and TAB I (the strain used to construct the library). DNA samples were digested with *EcoRI* and subjected to electrophoresis. The gel was Southern blotted, and the filter was hybridised with a pRMB2 probe. The resulting autoradiograph is shown in Figure 4.1.

This showed that the genomic insert of pRMB2 consisted of six *EcoRI* fragments, which were designated E1 to E6 in order of ascending size. The sizes of the fragments were calculated to be 1.1kb (E1), 2.5kb (E2), 2.7kb (E3), 4.7kb (E4), 5.1kb (E5) and 10.0kb (E6), giving a total insert size of 26.1kb. (Details of these calculations are given in Section 4.2.2). *EcoRI*-digested DNA from *B.pertussis* strains L84 I, Tohama I and TAB I hybridised to fragments E1 to E6 of pRMB2. In strain BP347 the E3 band was missing and replaced by an 8.4kb fragment. This implicated the E3 fragment of pRMB2 as part, at least, of the virulence regulatory *vir* locus. In the BP347 sample,



Figure 4.1.

Southern blot analysis of *EcoRI*-digested *B.pertussis* DNA probed with pRMB2.

Southern blot hybridisation was performed as described in Section 3.2.9. The probe was prepared by oligo-labelling purified pRMB2 DNA to high specific activity, by the method of Feinberg and Vogelstein (1984), as described in Section 3.2.10. On the left, E1 to E6 and pLAFR1, indicate the fragments comprising pRMB2. Numbers on the right refer to the fragment sizes (kb) of *HindIII*-cleaved  $\lambda$  DNA. (Hybridisation to E1 in genomic DNA samples was more clearly seen on a longer exposure.)

Lane 1: pRMB2

Lane 2: *B.pertussis* L84 I

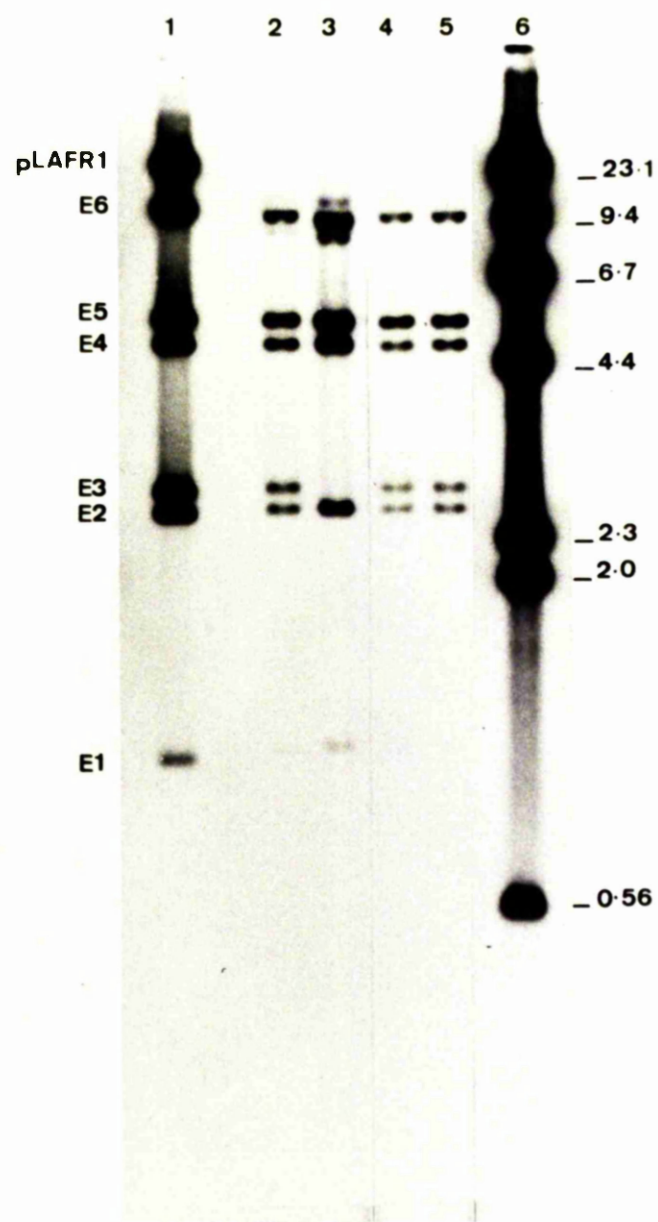
Lane 3: *B.pertussis* BP347

Lane 4: *B.pertussis* Tohama I

Lane 5: *B.pertussis* TAB I

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Lane 6: P-labelled *HindIII*-cleaved  $\lambda$  DNA



which contained an excess of DNA compared to the other samples, a faint high molecular weight band was also detected.

#### 4.1.2. Subcloning E3 from pRMB2 into vector pLAFR1.

The E3 fragment of pRMB2 was subcloned in the broad host-range vector pLAFR1, to give a construct which could subsequently be transferred from *E.coli* to BP347 for complementation analysis.

Plasmid pLAFR1 was digested with *EcoRI* and dephosphorylated using calf intestinal alkaline phosphatase (CIP), as described in Section 3.2.11. Plasmid pRMB2 was digested with *EcoRI* and subjected to electrophoresis through low melting-point agarose. E3 DNA was purified from a band excised from the gel. A ligation reaction containing 100ng of dephosphorylated pLAFR1 *EcoRI*-cleaved DNA and 100ng of E3 fragment DNA was set up. The reaction contained a 10-fold molar excess of E3 fragment DNA. As controls, 100ng of dephosphorylated pLAFR1 vector alone and 100ng of non-CIP treated pLAFR1 vector alone were ligated. Following ligation at room temp for 2h, samples were transformed into freshly prepared *E.coli* JM83 cells. To check the transformation efficiency, 1ng and 10ng samples of pUC19 plasmid DNA (Gibco BRL) were also transformed. Following transformation, samples were spread on nutrient agar containing the appropriate antibiotic. Competent *E.coli* JM83 cells were spread on nutrient agar containing Tc and Ap to check sensitivity to these antibiotics. The number of transformants obtained is given in Table 4.1.

*E.coli* JM83 cells were unable to grow on nutrient agar containing Tc or Ap so it was presumed that all colonies obtained were genuine transformants. As 1ng of pUC19 DNA gave 350 colonies, the transformation efficiency was calculated to be approximately  $3.5 \times 10^5$  transformants per

Table 4.1

Transformation of *E. coli* JM83 to create pDM3

---

DNA sample <sup>a</sup>	Number of colonies obtained <sup>b</sup>
<hr/>	
1. 100ng E3 + 100ng dephosphorylated pLAFR1	2
2. 100ng dephosphorylated pLAFR1	2
3. 100ng non-CIP treated pLAFR1	73
4. 1ng pUC19	350
5. 10ng pUC19	semi-confluent

---

a. Ligation samples (1, 2 and 3) 10μl and plasmid samples (4 and 5) 1μl were transformed with freshly-prepared competent *E. coli* JM83 cells, as described in Section 3.2.14.

b. Transformants were selected on nutrient agar containing Tc (for pLAFR1 and its derivatives) or Ap (for pUC19).



µg of DNA. Two colonies were obtained from ligated dephosphorylated pLAFR1 vector, which was considerably lower than the number obtained from ligated non-CIP treated vector (73). Only two transformants were obtained from the ligation mixture containing pLAFR1 vector and the E3 fragment. Plasmid DNA was prepared from each of the two colonies, digested with *EcoRI*, and subjected to electrophoresis. Visualisation of the ethidium bromide stained gel in u.v. light showed that one sample consisted of pLAFR1 vector alone, but the other consisted of pLAFR1 vector plus the E3 fragment. The latter was designated pDM3.

#### 4.1.3. Subcloning E3 from pRMB2 into vector pIC20H.

At this stage it was considered of interest to determine if the 2.7kb E3 fragment of pRMB2 could encode the product of the *vir* locus which would allow expression of virulence determinants not normally expressed in *E.coli*. For example, pRMB1 contains the *B.pertussis* adenylate cyclase (AC) genetic determinant cloned in pLAFR1, but no AC is expressed in *E.coli* (Brownlie *et al.*, 1988). To this end the E3 fragment was subcloned into the high copy-number plasmid vector pIC20H which would make it compatible with plasmids such as pRMB1.

Plasmid pIC20H was digested with *EcoRI*, and dephosphorylated. A ligation reaction containing 10ng of dephosphorylated vector DNA and 100ng of E3 DNA (purified as in Section 4.1.2) was set up. The molar ratio of insert to vector DNA was 10 to 1. As controls, ligations were also set up using 10ng of dephosphorylated pIC20H vector alone and 10ng of non-CIP treated pIC20H vector alone. Ligations were incubated at room temp for 2h. An aliquot from each ligation reaction was transformed into freshly prepared *E.coli* JM83 cells. Samples containing 1ng and 10ng of supercoiled

pIC20H plasmid DNA were also transformed to determine transformation efficiency. Transformation mixes were spread on nutrient agar supplemented with Ap, IPTG and X-gal. As a control, competent *E.coli* JM83 cells were also spread on a selection plate. The number of colonies obtained is shown in Table 4.2.

Plasmid pIC20H contains a polylinker, specifying 17 restriction sites, in the  $\beta$ -galactosidase  $\alpha$ -complementing gene fragment. Recombinant clones can be easily identified, as insertion of DNA molecules into the polylinker region results in inactivation of  $\beta$ -galactosidase  $\alpha$ -complementation, and colonies appear white on medium supplemented with IPTG plus X-gal.

As expected, *E.coli* JM83 was Ap sensitive. A 1ng sample of pIC20H DNA<sup>5</sup> resulted in 410 colonies, giving a transformation efficiency of  $4.1 \times 10^5$  transformants per  $\mu$ g of DNA. Ligated, non-CIP treated, *EcoRI*-cleaved pIC20H gave a 4-fold reduction in the number of colonies obtained per  $\mu$ g of DNA transformed when compared with supercoiled pIC20H. Dephosphorylated vector gave only 2 colonies, whereas non-CIP treated vector gave 103 colonies.

Transformation of the E3 fragment ligated with dephosphorylated *EcoRI*-cleaved pIC20H vector, resulted in 10 white and 3 blue colonies. The blue colonies were presumed to contain re-circularised vector DNA. Plasmid DNA was prepared from each of the 10 white colonies. Since pIC20H and the E3 fragment are approximately the same size, digestion with *EcoRI* would result in a doublet band. Therefore, to identify clones containing insert, *BamHI* was chosen because preliminary analysis had shown that the E3 fragment did not contain any *BamHI* sites, but a unique *BamHI* site is present in the polylinker of pIC20H. Plasmid samples were digested with *BamHI* and subjected to electrophoresis. Examination of the ethidium bromide stained gel in u.v. light, showed that two samples contained a band of 2.7kb,

Table 4.2.

Transformation of *E. coli* JM83 to create pDM30.

DNA sample <sup>a</sup>	Number of colonies obtained <sup>b</sup>
1. 10ng E3 + 1ng dephosphorylated pIC20H	10W, 3B
2. 1ng dephosphorylated pIC20H	2B
3. 1ng non-CIP treated pIC20H	103B
4. 1ng pIC20H	410B
5. 10ng pIC20H	semi-confluent B

a. Aliquots (1, 2 and 3) 1µl from ligated DNA samples and plasmid samples (4 and 5) 1µl were transformed with freshly-prepared competent *E. coli* JM83 cells, as described in Section 3.2.14.

b. Transformants were selected on nutrient agar containing Ap, IPTG and X-gal. 'W' denotes white, and 'B' denotes blue colony colour.

presumed to be linearised pIC20H vector, but the remaining eight samples contained a band of 5.4kb, presumed to be pIC20H containing the E3 fragment. The orientation of the E3 fragment with respect to the *lac* promoter was not determined. This construct was designated pDM30.

#### 4.1.4. Hybridisation of E3 to BP347 DNA.

When *EcoRI*-digested DNA from BP347 was probed with pRMB2, the 2.7kb band was missing, and a larger band was present (Section 4.1.1). It was presumed that this band was the 2.7kb *EcoRI* fragment containing a copy of transposon Tn5. To determine if this was actually the case, *EcoRI*-digested DNA from pRMB2 and from *B.pertussis* strains L84 I, BP347, Tohama I and TAB I was Southern blotted, and hybridised with a pDM3 probe (Figure 4.2).

As expected, pDM3 hybridised to pLAFR1 and the 2.7kb E3 fragment of *EcoRI*-digested pRMB2. The pDM3 probe hybridised to a 2.7kb *EcoRI* fragment in DNA from *B.pertussis* strains L84 I, Tohama I and TAB I. With BP347 DNA, the E3 fragment of pDM3 hybridised to an 8.4kb fragment. This confirmed that the 8.4kb fragment of BP347 was the 2.7kb *EcoRI* fragment containing a copy of Tn5.

#### 4.1.5. Complementation of BP347 by pDM3.

Clone pDM3 was transferred by conjugation from *E.coli* JM83 to *B.pertussis* BP347, using a tri-parental mating system which included a helper plasmid (pRK2013). Donor cultures of *E.coli* HB101 (pLAFR1) and *E.coli* DH1 (pRMB2) were included as negative and positive controls respectively. Conjugation mixes were incubated overnight at 35°C. The cells were then transferred to selective BG agar. Donor, helper and recipient strains used as controls were unable to grow on this selective medium.

Figure 4.2.

Southern blot analysis of *EcoRI*-digested *B.pertussis* DNA probed with pDM3.

The blot was hybridised with oligo-labelled pDM3 plasmid. E3 and pLAFR1, indicate the fragments of pRMB2 to which pDM3 hybridised. Numbers on the right refer to the fragment sizes (kb) of *HindIII*-cleaved  $\lambda$  DNA.

Lane 1: pRMB2

Lane 2: *B.pertussis* L84 I

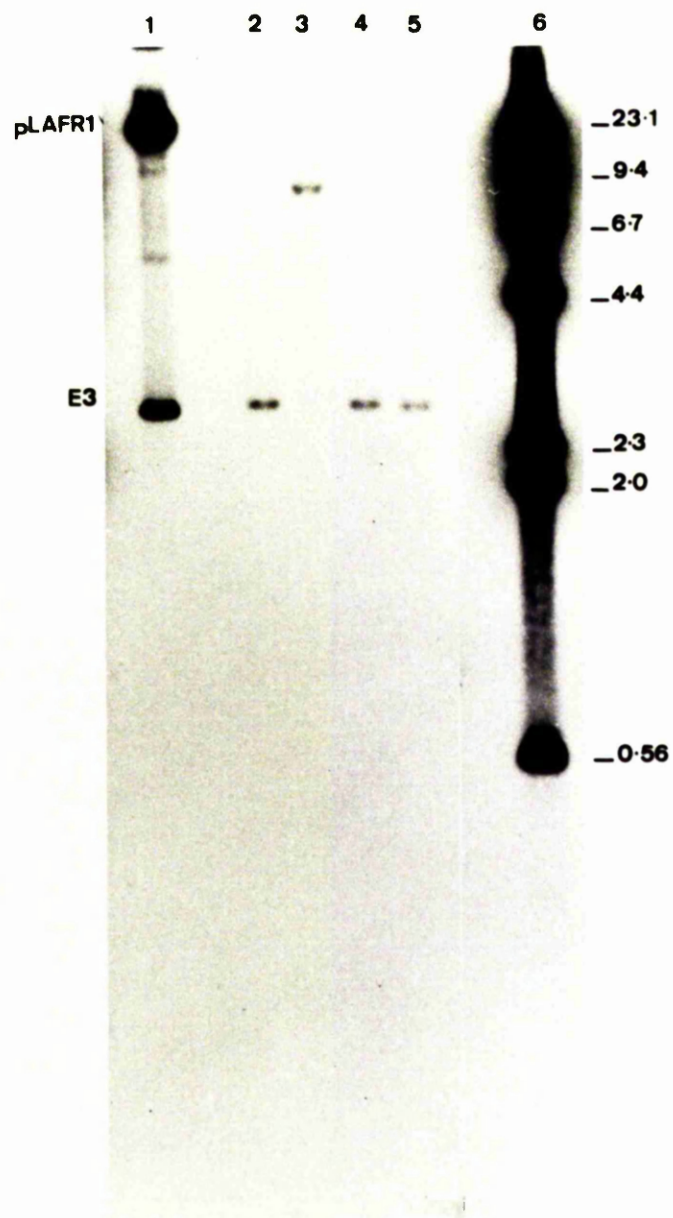
Lane 3: *B.pertussis* BP347

Lane 4: *B.pertussis* Tohama I

Lane 5: *B.pertussis* TAB I

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Lane 6: P-labelled *HindIII*-digested  $\lambda$  DNA



After incubation at 35°C for 3-4 days, all plates supported semi-confluent growth of transconjugants. The morphology of transconjugant cells was checked by Gram staining and found to be like that of *B.pertussis*. As expected, BP347 (pLAFR1) transconjugant colonies were all non-haemolytic, and BP347 (pRMB2) transconjugants were all haemolytic. However, although the majority of BP347 (pDM3) colonies were non-haemolytic, approximately 1% of colonies showed haemolysis. Non-haemolytic BP347 (pDM3) transconjugants were designated BP347 (pDM3) H-, and haemolytic BP347 (pDM3) transconjugants were designated BP347 (pDM3) H+.

To establish if the above results were in fact typical, the conjugation experiment was repeated with *E.coli* JM83 (pDM3) donor cultures set up in duplicate. Conjugation mixes were incubated at 35°C for only 8h (instead of overnight, as above) before being transferred to selective BG agar.

As before, BP347 (pLAFR1) transconjugants were all non-haemolytic and BP347 (pRMB2) transconjugants all showed haemolysis. However, both BP347 (pDM3) selective agar plates supported growth of only non-haemolytic colonies.

To determine whether pDM3 had restored expression of any other virulence-associated factors to BP347, *B.pertussis* strains BP347, BP347 (pLAFR1), BP347 (pRMB2), BP347 (pDM3) H-, and BP347 (pDM3) H+ were assayed for production of AC and FHA. The phenotype of each strain was also examined on Congo red agar. Results are shown in Table 4.3.

*B.pertussis* strains BP347 and BP347 (pLAFR1) were negative, whereas BP347 (pRMB2) was positive for all activities tested. Non-haemolytic BP347 (pDM3) H- was also negative for adenylate cyclase and filamentous haemagglutinating activities, and was unable to bind Congo red. Conversely, BP347 (pDM3) H+ was positive for expression of AC and FHA, and was able to

Table 4.3.

Complementation of BP347 by pDM3

<i>B. pertussis</i> strain	Phenotype <sup>a</sup>			
	HLY	AC	FHA	CR
BP347	-	-	-	-
BP347 (pLAFR1)	-	-	-	-
BP347 (pRMB2)	+	+	+	+
BP347 (pDM3) H-	-	-	-	-
BP347 (pDM3) H+	+	+	+	+

a. HLY = haemolysin, AC = adenylate cyclase, FHA = filamentous haemagglutinin, CR = Congo red binding.



bind Congo red.

Further analysis of BP347 (pDM3) H<sup>+</sup> transconjugants is given in Section 4.1.11, which indicates that they arose by homologous recombination in the *vir* region. On the assumption that the E3 fragment does not contain all of the *vir* locus, the ability of pDM30 (Section 4.1.3) to *trans*-activate the cloned *B.pertussis* AC genetic determinant (pRMB1) in *E.coli* was not investigated at this stage.

#### 4.1.6. Subcloning and complementation analysis of *Sau3A* fragments of pRMB2.

Subclones containing an insert of approximately 6kb of DNA were constructed after partial digestion of pRMB2 with *Sau3A*, and ligation of fragments into broad host-range vectors to enable their subsequent transfer to BP347.

Initially plasmid pRK291 was used as the cloning vector. This broad host-range plasmid is a derivative of pRK290 in which the *BglIII* site has been converted to *BamHI*. Plasmid pRK291 was digested with *BamHI* and dephosphorylated.

Insert DNA was prepared by partially digesting pRMB2 DNA with *Sau3A*. A pilot digest was performed as described in Section 3.2.5. Samples were removed after 2.5, 5, 7.5, 10 and 12.5 min of incubation with the enzyme, and analysed by electrophoresis. The ethidium bromide stained gel is shown in Figure 4.3. As the period of incubation lengthened, pRMB2 became progressively more digested.

The partial digest was then repeated using a 5 min incubation period, since this was shown by the pilot reaction to result in the largest proportion of fragments of around 6kb. The sample of pRMB2 DNA, partially digested with *Sau3A*, was subjected to electrophoresis through low melting-

Figure 4.3.

Electrophoresis of pRMB2 partially digested with *Sau3A*.

A pilot digest of pRMB2 with *Sau3A* was performed as described in Section 3.2.5. Samples were removed at intervals after initiation of digestion, and analysed by electrophoresis (lanes 1 to 5). Fragments of around 6kb are contained within the portion of the gel between the horizontal lines.

The *Sau3A* digest of pRMB2 was repeated (using a 5 min incubation period), and lane 8 shows an aliquot of size-selected *Sau3A* fragments after purification from low melting-point agarose. *HindIII*-digested  $\lambda$  DNA was included on both gels to give molecular weight standards of 23.1kb, 9.4kb, 6.7kb, 4.4kb, 2.3kb, 2.0kb and 0.56kb.

Lane 1: 2.5 min sample

Lane 2: 5 min sample

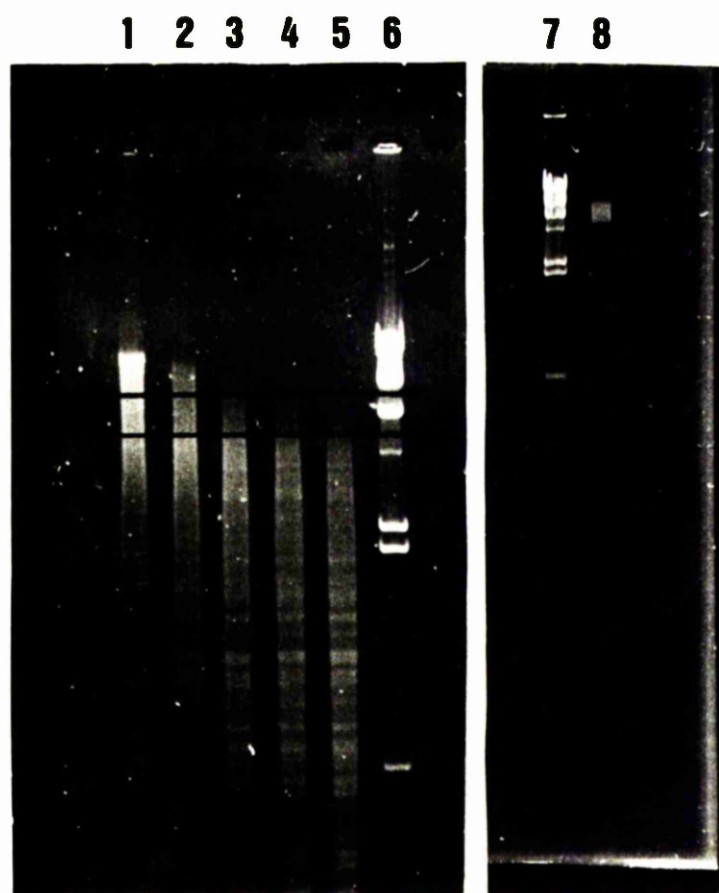
Lane 3: 7.5 min sample

Lane 4: 10 min sample

Lane 5: 12.5 min sample

Lanes 6 and 7: Molecular weight standards

Lane 8: purified *Sau3A* fragments of pRMB2



point agarose, and DNA was recovered from a gel slice cut to include fragments of around 6kb. Electrophoresis of a small aliquot of the purified DNA showed that the *Sau3A* fragments ranged in size from approximately 5kb to 9kb (Figure 4.3).

*BamHI*-cleaved dephosphorylated pRK291 vector 100ng and *Sau3A* fragments 100ng were ligated together overnight, and transformed into competent *E.coli* JM83. Cells harbouring plasmid were selected on nutrient agar containing Tc. Eight colonies were obtained. However, *EcoRI*-digestion of plasmid DNA purified from these colonies showed that they all contained re-circularised pRK291. Transformation efficiency was tested by transforming *E.coli* JM83 with 1ng  $\mu$ UC19 plasmid DNA (Gibco BRL) and calculated to be  $5 \times 10^5$  transformants per  $\mu$ g DNA.

Since cloning into pRK291 vector proved difficult, vector pRK310 was tried as an alternative. Plasmid pRK310 is also derived from pRK290, and contains a polylinker sequence on a lacZ ( $\alpha$ ) peptide coding region. This was advantageous in that it allowed immediate visual identification of recombinant clones as white colonies on medium containing IPTG plus X-gal, and also the restriction enzyme sites on either side of the *BamHI* site enabled insert DNA to be cut out of recombinant clones.

Plasmid pRK310 was digested with *BamHI* and dephosphorylated. To ensure that a sufficient number of recombinant clones would be obtained for the purpose of screening for an active *vir* locus, a number of ligation reactions containing various concentrations of dephosphorylated *BamHI*-cleaved pRK310 vector and purified *Sau3A* fragments of pRMB2 were set up, as shown in Table 4.4. Each ligation reaction contained a molar excess of insert DNA. After ligation overnight, samples were transformed with freshly-prepared competent *E.coli* JM83 cells. As controls, *E.coli* JM83 was

Table 4.4.

Transformation of *E. coli* JM83 to create pRK310 recombinant clones

DNA sample <sup>a</sup>	number of transformants obtained <sup>b</sup>
1. 50 ng vector + 50 ng insert	6 W
2. 100 ng vector + 100 ng insert	9 W
3. 50 ng vector + 100 ng insert	2 W
4. 50 ng vector + 150 ng insert	4 W
5. 100 ng vector + 50 ng insert	19 W
6. 250 ng vector + 100 ng insert	8 W
7. 10 ng pRK310	15 B
8. 100 ng pRK310	approximately 200 B
9. 10 ng pIC20H	semi-confluent B
a. Ligation samples (1 to 6) 10 µl containing <i>Bam</i> HI-digested pRK310 vector and <i>Sau</i> 3a fragments of pRMB2 at various concentrations, and plasmid samples (7 to 9) 1 µl were transformed with competent <i>E. coli</i> JM83 cells as described in Section 3.2.14.	
b. Transformants were selected on nutrient agar containing Tc, IPTG and X-gal (for pIC20H). 'W' denotes white, and 'B' denotes blue colony colour.	

also transformed with 10ng and 100ng of pRK310 plasmid, and 10ng of pIC20H plasmid. Transformants were selected on nutrient agar supplemented with the appropriate antibiotic, IPTG and X-gal. The number of transformants obtained is given in Table 4.4.

Transformation with 10ng of pRK310 resulted in 15 blue colonies, and 100ng of pRK310 gave approximately 200 blue colonies. The transformation efficiency was therefore  $1.5 - 2.0 \times 10^3$  transformants per  $\mu\text{g}$  pRK310 DNA. However, transformation with 10ng of pIC20H resulted in a semi-confluent lawn of blue colonies. Therefore, the transformation efficiency of pIC20H DNA was considerably higher than that of the larger plasmid pRK310. Transformation of *E.coli* JM83 with the ligation reactions resulted in a total of 48 white colonies (numbered 1 to 48) presumed to be recombinant clones.

To determine whether any of the pRK310 recombinant clones contained the *vir* locus, clones were transferred by conjugation to BP347. All 48 clones were inoculated, in duplicate, into the wells of two Cooke microtitre trays containing nutrient broth supplemented with Tc. *E.coli* DH1 (pRMB2) and *E.coli* HB101 (pRK310) donor cultures were also set up in duplicate to represent positive and negative controls respectively. Using a tri-parental mating system which included the use of a helper plasmid (pRK2013), conjugation was performed as described in Section 3.2.15, each conjugation mix being finally inoculated onto selective BG agar using the multi-point inoculator.

BP347 (pRMB2) transconjugants were haemolytic and BP347 (pRK310) transconjugants were non-haemolytic. All BP347 transconjugants containing pRK310 recombinant clones were negative for haemolytic activity.

To calculate the average insert size of the pRK310 recombinant clones,

plasmid DNA was prepared from six of them (numbers 1, 9, 17, 25, 33 and 41). The enzymes *HindIII* and *EcoRI* were chosen for analysis of plasmid DNA. Restriction sites for these enzymes are located at the ends of the polylinker sequence, and would enable insert DNA to be cut out of recombinant clones. An additional *EcoRI* restriction site is contained within the pRK310 vector. DNA from pRK310 recombinant clones and plasmid pRK310 was digested with both *HindIII* and *EcoRI* and subjected to electrophoresis (Figure 4.4). Band migration distances were measured from a photograph of the ethidium bromide stained gel by automated densitometry and molecular sizes of fragments were estimated by the procedure of Plikaytis *et al.* (1986), as described in Section 3.2.7.

Digestion of pRK310 with both *HindIII* and *EcoRI* resulted in production of two fragments of approximately 11.0kb and 9.4kb. As the *BamHI* site (into which the *Sau3A* fragments of pRMB2 were cloned) is located within the polylinker, digestion of the recombinant clones yielded the two vector fragments plus insert DNA. The sizes of the inserts were determined as 3.7kb (clone 1), 8.3kb (clone 9), 6.3kb (clone 17), 3.1kb (clone 25), 5.5kb (clone 33) and 5.5kb (clone 41). The average insert size for the six clones analysed was 5.4kb.

The cloning of *Sau3A* fragments of pRMB2 into the broad host-range vectors, with subsequent transfer of recombinant clones to BP347, was a laborious procedure and a more direct method of obtaining a subclone of pRMB2 containing the *vir* locus was sought.

Figure 4.4.

Electrophoresis of pRK310 recombinant clones digested with both *HindIII* and *EcoRI*.

DNA from pRK310 recombinant clones and plasmid pRK310 was digested with both *HindIII* and *EcoRI*, and subjected to electrophoresis. Band migration distances were measured from a photograph of the ethidium bromide stained gel using a soft laser-scanning densitometer, and molecular sizes (shown in brackets below) of fragments comprising pRK310 recombinant clones were estimated by a modification of the method of Plikaytis *et al.* (1986) (see Section 3.2.7), using *HindIII*-digested  $\lambda$  DNA as molecular weight standards. A digitised version of the ethidium bromide stained gel is shown here.

Lane 1: clone 1 (11.0kb, 9.4kb, 3.7kb)

Lane 2: clone 9 (11.0kb, 9.4kb, 4.6kb, 3.7kb)

Lane 3: clone 17 (11.0kb, 9.4kb, 6.3kb)

Lane 4: clone 25 (11.0kb, 9.4kb, 3.1kb)

Lane 5: clone 33 (11.0kb, 9.4kb, 5.5kb)

Lane 6: clone 41 (11.0kb, 9.4kb, 3.3kb, 2.2kb)

Lane 7: pRK310 (11.0kb, 9.4kb)



kb

30 -

20 -

10 -

5 -

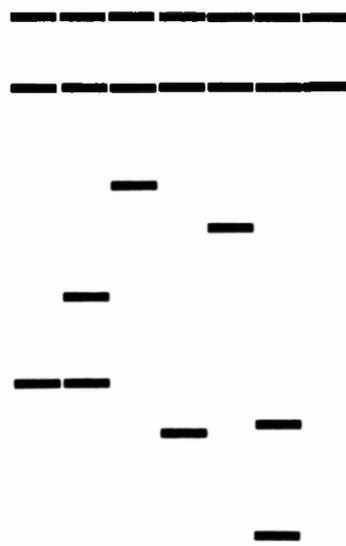
3 -

2 -

1 -

0.5 -

1 2 3 4 5 6 7



#### 4.1.7. Preliminary analysis of the location of E3 within pRMB2.

The E3 fragment, known to be essential to *vir*, was used to probe various restriction digests of pRMB2 in order to identify a larger fragment which might contain a functional *vir* locus.

The restriction enzymes chosen were *Bam*HI, *Bgl*III, *Pst*I and *Sac*I. Plasmid pRMB2 was digested with each of these enzymes, and also with *Eco*RI as a control. Digested DNA was subjected to electrophoresis together with *Bam*HI-digested pDM30 (Figure 4.5a). All pRMB2 digests showed fragments of greater than 2.7kb which could have contained a functional *vir* locus.

The gel was Southern blotted, and the filter was hybridised with a pDM30 probe (Figure 4.5b). Plasmid pDM30 hybridised to itself, and to the 2.7kb *Eco*RI fragment of pRMB2, confirming that clone pDM30 contains the E3 fragment. Plasmid pDM30 hybridised to a 2.8kb fragment, and also faintly to a larger fragment, in *Pst*I-digested pRMB2. In DNA samples digested with *Bam*HI, *Bgl*III and *Sac*I, pDM30 hybridised to the largest fragment in each instance, consisting of pLAFR1 vector DNA and the terminal portions of the genomic insert of pRMB2. Each of these three fragments potentially contained sufficient sequence to encode *vir*. The *Bam*HI fragment was chosen for analysis.

#### 4.1.8. Identification of the site of Tn5 insertion in BP347 with respect to *Bam*HI fragments.

*Bam*HI-digested DNA from pRMB2 and *B.pertussis* strains L84 I, BP347, Tohama I and TAB I was Southern blotted. The filter was hybridised with oligo-labelled pRMB2 plasmid in order to determine the site of Tn5 insertion in BP347 with respect to *Bam*HI fragments (Figure 4.6a). Plasmid pRMB2 consisted of fragments of 1.4kb, 2.4kb, 2.7kb, 2.9kb, 4.8kb and a

Figure 4.5.

**Electrophoresis and Southern blot analysis of restriction digests of pRMB2.**

Plasmid pRMB2 DNA which had been digested with various restriction enzymes was subjected to electrophoresis, together with *Bam*HI-digested pDM30 DNA. The ethidium bromide stained gel is shown in (A). The gel was Southern blotted and hybridised with oligo-labelled pDM30 plasmid (B). The corresponding bands to which pDM30 hybridised have been marked with an asterisk in (A). The sizes (kb) of *Eco*RI-digested pRMB2 fragments are given on the left.

Lane 1: *Bam*HI-digested pRMB2

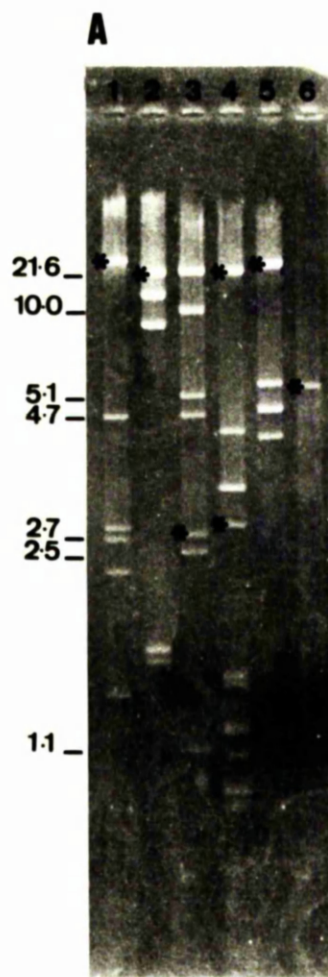
Lane 2: *Bgl*III-digested pRMB2

Lane 3: *Eco*RI-digested pRMB2

Lane 4: *Pst*I-digested pRMB2

Lane 5: *Sac*I-digested pRMB2

Lane 6: *Bam*HI-digested pDM30



**B**

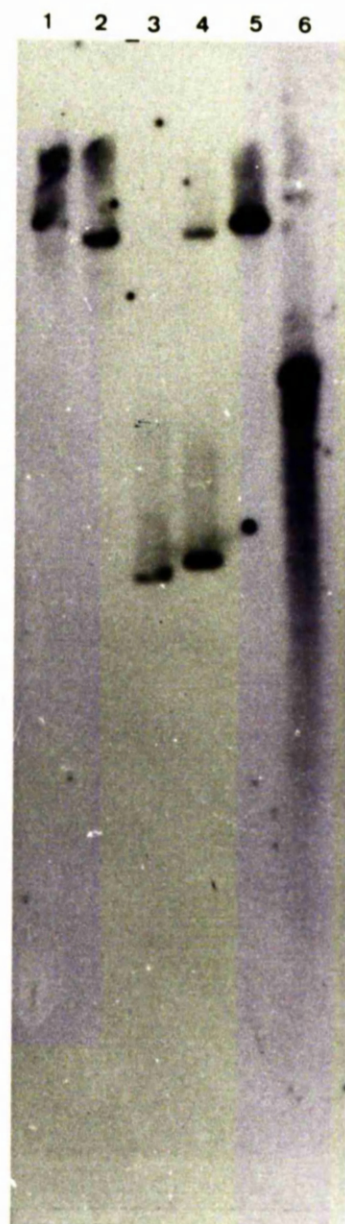


Figure 4.6.

Southern blot analysis of *Bam*HI-digested *B.pertussis* DNA probed with pRMB2 and E3.

The nitrocellulose filter was hybridised with oligo-labelled pRMB2 plasmid (A). Residual radioactivity was removed, as described in Section 3.2.9. The filter was rehybridised with a probe made by oligo-labelling E3 fragment which had been purified from a gel by centrifugation through a SPIN-X filter unit (B). Numbers on the left refer to fragment sizes (kb) of *Hind*III-cleaved  $\lambda$  DNA. To the right of (A), numbers B1 to B6 indicate the fragments observed in pRMB2.

32

Lane 1: P-labelled *Hind*III-cleaved  $\lambda$  DNA

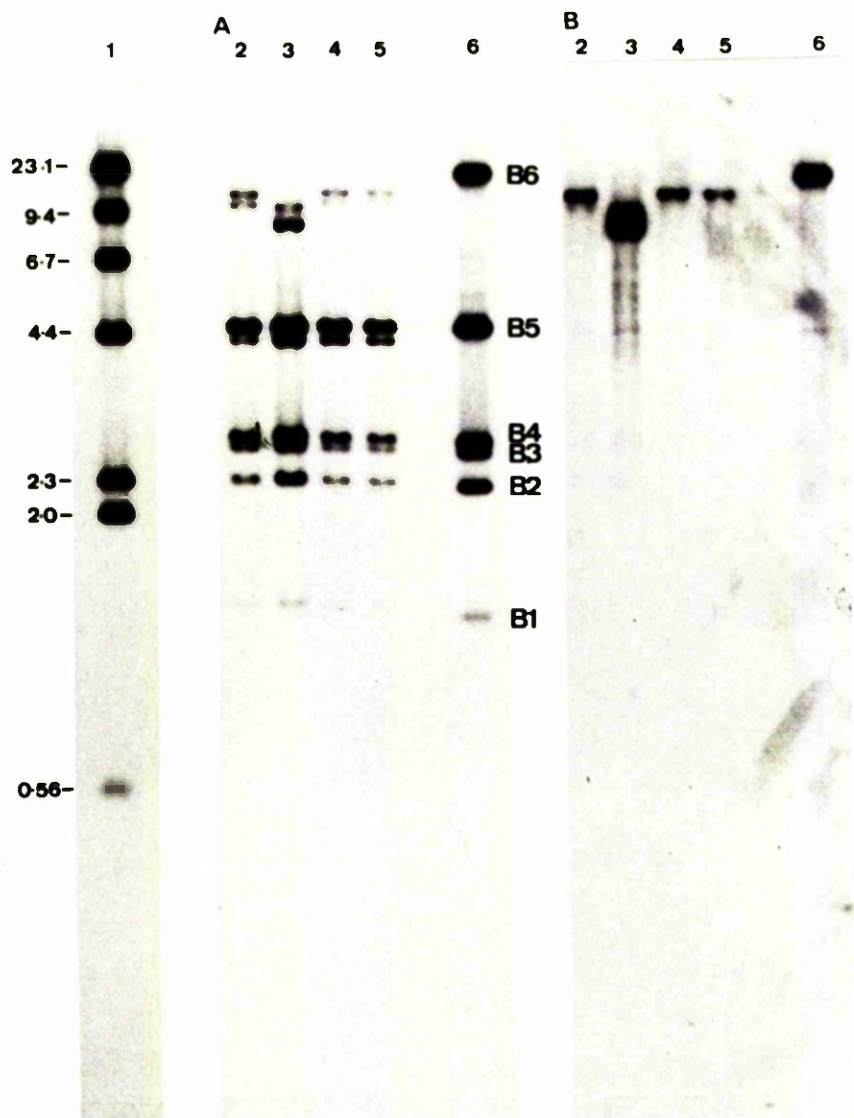
Lane 2: *B.pertussis* L84 I

Lane 3: *B.pertussis* BP347

Lane 4: *B.pertussis* Tohama I

Lane 5: *B.pertussis* TAB I

Lane 6: pRMB2



large fragment consisting of pLAFR1 vector and the terminal portions of the genomic insert. These fragments were designated B1 to B6 in order of ascending size. (Details of calculations of fragment sizes are given in Section 4.2.2).

The B1 to B5 fragments of the probe hybridised to corresponding fragments in *Bam*HI-digested genomic DNA for all samples. In samples of *B.pertussis* strains L84 I, Tohama I and TAB I the B6 fragment of the probe hybridised to two *Bam*HI genomic fragments, of approximately 12kb and 14kb. However, for strain BP347, the 14kb band was missing, and exhibited instead was a band of approximately 9kb. Since transposon Tn5 contains a *Bam*HI site, this 9kb fragment was presumed to be a result of Tn5 insertion into the larger of the two genomic fragments showing homology to the *Bam*HI to *Eco*RI ends of the genomic insert of pRMB2. Plasmid pRMB2 also hybridised weakly to a band of approximately 4.2kb in all *B.pertussis* samples.

The more specific E3 fragment of pRMB2 was then used as a probe. The filter was stripped to remove residual radioactivity, and rehybridised with oligo-labelled E3 fragment (Figure 4.6b). E3 hybridised to the B6 fragment of pRMB2, as previously described in Section 4.1.7. For *B.pertussis* strains L84 I, Tohama I and TAB I, E3 hybridised to the larger of the two *Bam*HI fragments of genomic DNA which showed homology to the *Bam*HI to *Eco*RI' ends of pRMB2. For BP347, E3 hybridised to the 9kb fragment. Since the E3 fragment hybridised to the site of Tn5 insertion in BP347 (Section 4.1.4), these data implied that the larger of the two *Bam*HI genomic fragments contained all, or part of, the *vir* locus.

#### 4.1.9. Construction of clone pDM1.

Since the E3 fragment alone was unable to complement the Vir- mutation of BP347 (Section 4.1.5), the B6 fragment of pRMB2 (shown by hybridisation studies to include E3, Sections 4.1.7 and 4.1.8) was isolated and ligated to give a construct which could subsequently be transferred to BP347.

Plasmid pRMB2 was digested with *Bam*HI and subjected to electrophoresis through low melting-point agarose. A band containing the B6 fragment was excised. This fragment, consisting of pLAFR1 and the *Bam*HI to *Eco*RI terminal portions of the genomic insert of pRMB2, was purified from the gel slice. Fragment B6 100ng was ligated at room temp overnight, and transformed into competent *E.coli* JM83. 1ng of pUC19 was also transformed. Transformants were selected on nutrient agar supplemented with the appropriate antibiotic. The transformation efficiency was  $5.7 \times 10^5$  transformants per  $\mu$ g of pUC19 DNA. Sixteen colonies were obtained from transformation by ligated B6 DNA, six of which were used for preparation of plasmid DNA. Electrophoresis of *Bam*HI-digested plasmid DNA showed that all six clones contained B6. This construct was designated pDM1.

Figure 4.7 shows electrophoresis of *Eco*RI-digested pRMB2, pDM1, pDM3 and pLAFR1, together with *Bam*HI-digested pRMB2 and pDM1. Digestion of pRMB2 with *Eco*RI produced fragments E1 to E6 plus pLAFR1 vector. *Bam*HI-digestion of pRMB2 produced six fragments (B1 to B6). *Eco*RI-digestion of pDM1 showed that it consisted of pLAFR1 vector and 4 fragments, i.e. E1, E2, E3 plus a fragment made by ligation of the two *Eco*RI to *Bam*HI fragments of pRMB2. *Bam*HI-digestion of pDM1 produced the B6 fragment. *Eco*RI-digestion of pDM3 produced pLAFR1 plus the E3 fragment, and *Eco*RI-digestion of pLAFR1 gave a single band.



Figure 4.7.

Restriction enzyme profiles of clones pRMB2, pDM1 and pDM3.

Ethidium bromide stained gel showing samples of digested plasmid DNA. Samples of *EcoRI*-digested pRMB2 and pDM1 contain a band of 1.1kb, which is barely visible in this photograph. Numbers on the right refer to the fragment sizes (kb) of *HindIII*-cleaved  $\lambda$  DNA.

Lane 1: *EcoRI*-digested pRMB2

Lane 2: *BamHI*-digested pRMB2

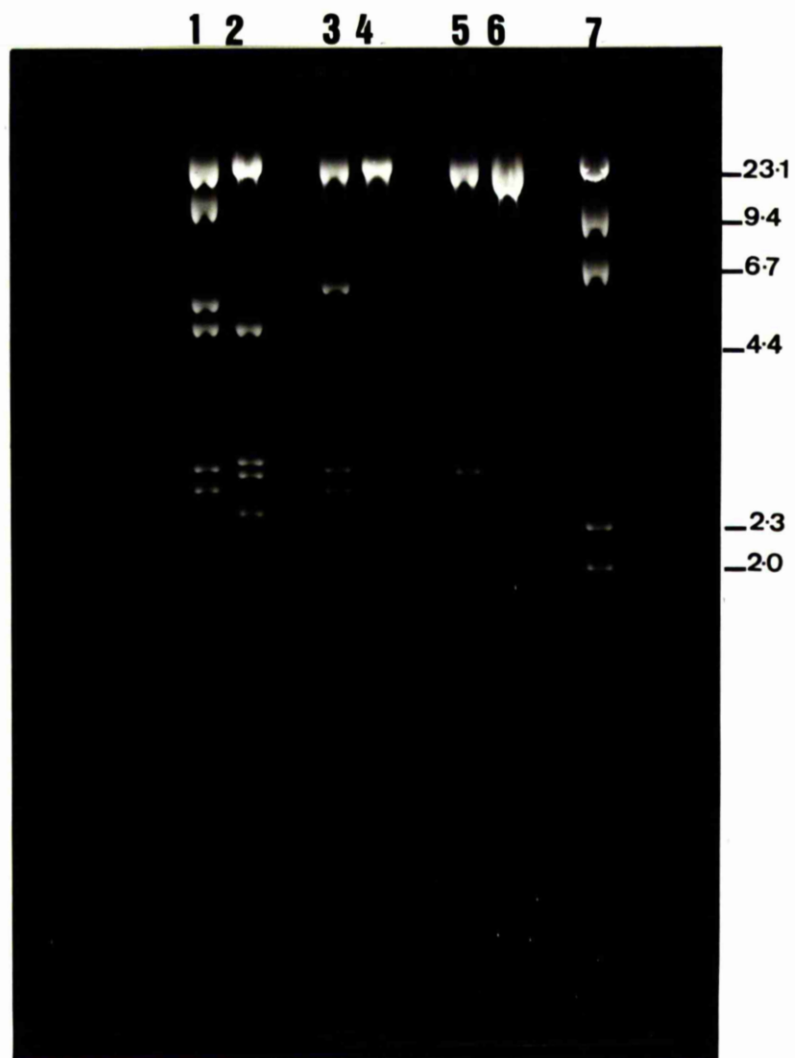
Lane 3: *EcoRI*-digested pDM1

Lane 4: *BamHI*-digested pDM1

Lane 5: *EcoRI*-digested pDM3

Lane 6: *EcoRI*-digested pLAFR1

Lane 7: *HindIII*-digested  $\lambda$  DNA



#### 4.1.10. Complementation of BP347 by pDM1.

The ability of pDM1 to complement the Vir- mutation of BP347 and restore a virulent phenotype to the strain was investigated. Clone pDM1 was transferred by conjugation from *E.coli* JM83 to BP347, using a tri-parental mating system. *E.coli* DH1 (pRMB2) and *E.coli* HB101 (pLAFR1) were included as positive and negative controls respectively. Conjugation mixes were incubated at 35°C for 8h. Cells were then transferred to selective BG agar. Donor, helper and recipient strains used as controls were unable to grow on selective BG agar. Following incubation at 35°C for 3 to 4 days, all plates supported semi-confluent growth of transconjugants. BP347 (pRMB2) transconjugants were haemolytic and BP347 (pLAFR1) transconjugants were non-haemolytic. All BP347 (pDM1) transconjugants were positive for haemolytic activity.

To determine if pDM1 restored the virulent phenotype to BP347, BP347 (pDM1) was also assayed for expression of AC, FHA, PT, HLT and X-OMPs. Included in the assays were *B.pertussis* strains Tohama I, TAB I and BP347 (pRMB2) as positive controls, and strain BP347 as a negative control. All strains were grown on BG agar (Tc was included in the medium for growth of strains harbouring plasmids), and haemolytic activity was assessed prior to growth being harvested into PBS for assay of other virulence-associated factors. Results are summarized in Table 4.5.

Production of AC was assayed by the development of a blue colour from X-gal in the wells of a microtitre tray. This assay relies on expression of  $\beta$ -galactosidase in *E.coli* G806 ( $\Delta$  cya) which is dependent on cAMP produced by *B.pertussis* AC. As a control, a reaction was set up to which no *B.pertussis* sample was added. Also included in the assay was a sample of *B.pertussis* L84 I. Results are shown in Figure 4.8. A sample of cAMP was

Table 4.5.

Complementation of BP347 by pDM1

<i>B. pertussis</i> strain	Phenotype <sup>a</sup>					
	HLY	AC	FHA	PT	HLT	X-OMPs
Tohama I	+	+	+	+	+	+
TAB I	+	+	+	+	+	+
BP347	-	-	-	-	-	-
BP347 (pRMB2)	+	+	+	+	+	+
BP347 (pDM1)	+	+	+	+	+	+

a. HLY = haemolysin, AC = adenylate cyclase, FHA = filamentous haemagglutinin, PT = pertussis toxin, HLT = heat-labile toxin, X-OMPs = X-mode specific envelope polypeptides.

Figure 4.8.

Assay for production of AC by *B.pertussis* BP347 (pDM1).

Production of AC was assayed by the development of a blue colour from X-gal in the wells of a microtitre tray, as described in Section 3.3.11. Samples were set up in triplicate (rows 1, 2 and 3). Numbers on the left refer to row numbers. Row 4 contains a sample of cAMP, diluted 10-fold from 10mM to 1nM as indicated by the arrow.

A: control with no *B.pertussis* sample added

B: *B.pertussis* L84 I

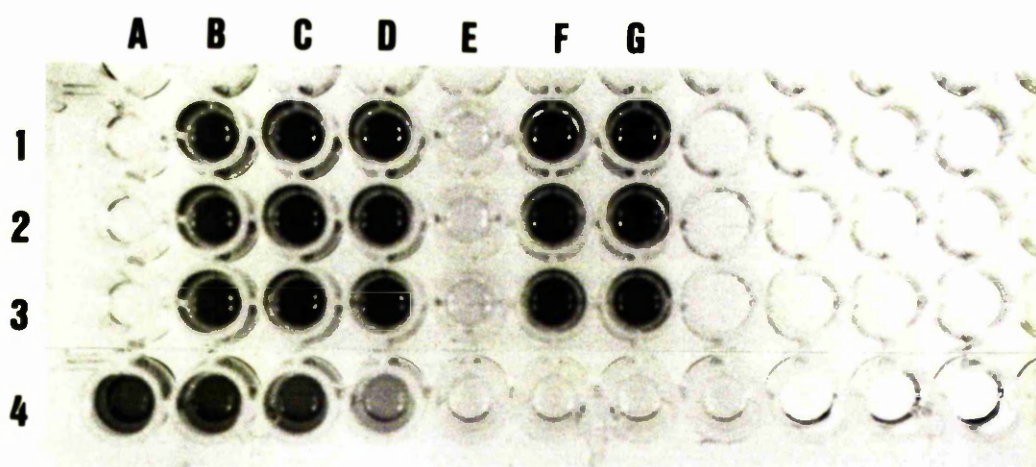
C: *B.pertussis* Tohama I

D: *B.pertussis* TAB I

E: *B.pertussis* BP347

F: *B.pertussis* BP347 (pDM1)

G: *B.pertusis* BP347 (pRMB2)



diluted 10-fold from 10mM to 1nM. A blue colour was detected at concentrations of 10 $\mu$ M and above. Samples of *B.pertussis* strains L84 I, Tohama I, TAB I, 347 (pRMB2) and 347 (pDM1) were positive for AC production, whereas strain BP347 was negative. There was no development of blue colour in the reaction which did not contain a *B.pertussis* sample.

FHA production was measured by haemagglutination of washed horse erythrocytes (Figure 4.9). Haemagglutination was found in *B.pertussis* strains Tohama I, TAB I, 347 (pRMB2) and BP347 (pDM1), but not in strain BP347.

Production of HLT and PT was determined in mice. Apart from strain BP347, all *B.pertussis* samples were positive for both HLT and PT activities.

The *B.pertussis* whole-cell samples were examined using SDS-PAGE to assay for the 28kD and 30kD X-OMPs (Figure 4.10). Only strain BP347 lacked the X-mode specific envelope polypeptide bands.

The restoration of expression of virulence-associated factors to BP347 by clone pDM1 implied that a functional *vir* locus was contained within pDM1.

#### 4.1.11. Stability of BP347 transconjugants in the absence of tetracycline selection.

The work described in this section was performed to investigate whether expression of virulence-associated properties in BP347 transconjugants had been restored as a result of the plasmid encoded *vir* locus acting *in-trans*, or as a result of recombination events which had occurred between plasmid and chromosomal DNA.

Figure 4.9.

Assay for production of FHA by *B.pertussis* BP347 (pDM1).

Production of FHA was assayed as described in Section 3.3.5. *B.pertussis* samples were serially diluted 2-fold from 50µg to 0.2µg protein per well (columns 2 to 10). As a control, column 11 contains PBS + erythrocytes.

Row B: *B.pertussis* Tohama I

Row C: *B.pertussis* TAB I

Row D: *B.pertussis* BP347

Row E: *B.pertussis* BP347 (pDM1)

Row F: *B.pertussis* BP347 (pRMB2)



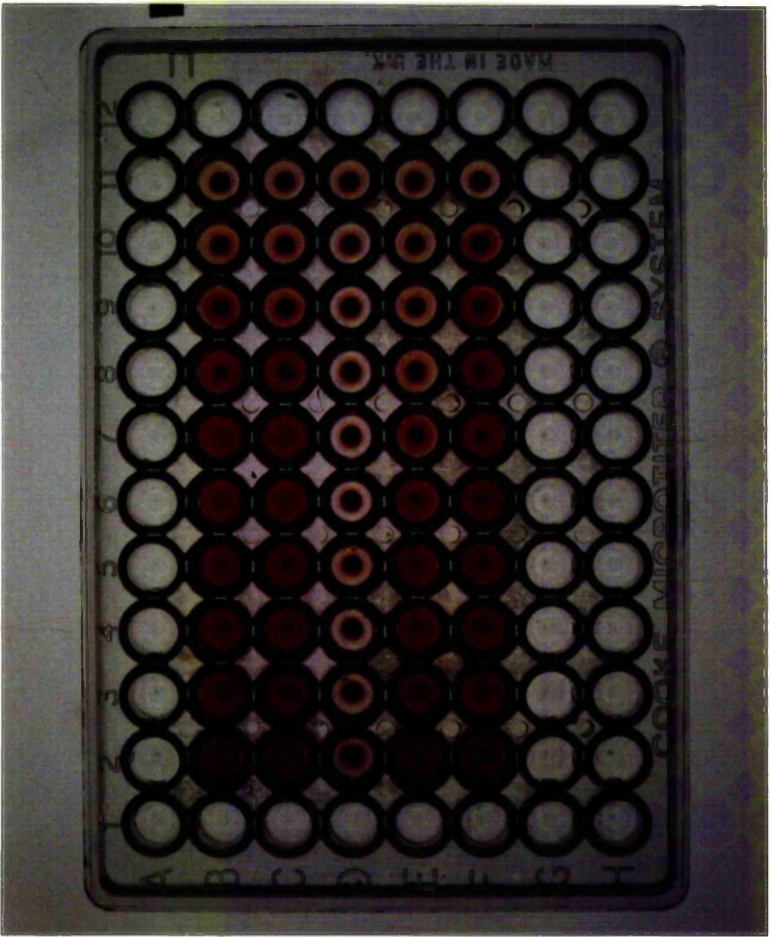


Figure 4.10.

SDS-PAGE analysis of *B.pertussis* BP347 (pDM1).

Whole-cell samples were treated and subjected to electrophoresis as described in Sections 3.3.1 and 3.3.2. Protein bands were visualised by Coomassie Blue staining. Numbers on the left refer to the sizes (kD) of molecular weight markers. Arrows mark the positions of the 28kD and 30kD X-OMPs.

Lanes 1 & 7: Molecular weight markers (SDS-6, Sigma)

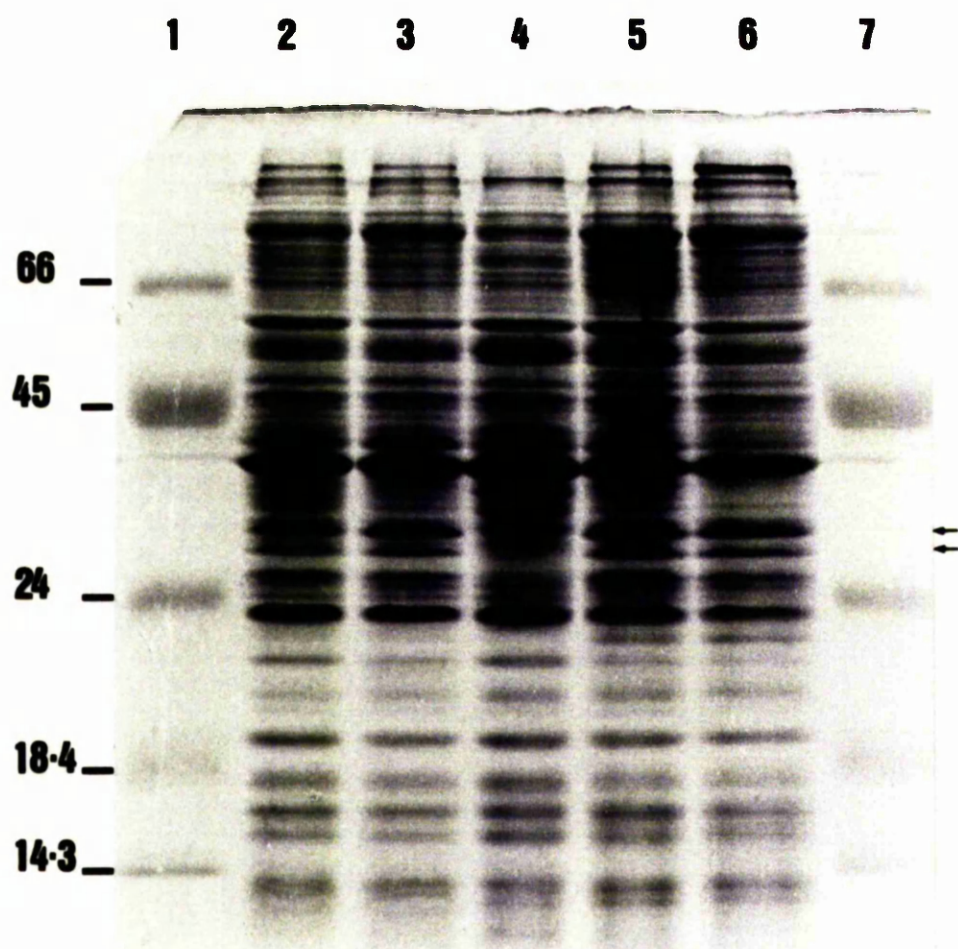
Lane 2: *B.pertussis* Tohama I

Lane 3: *B.pertussis* TAB I

Lane 4: *B.pertussis* BP347

Lane 5: *B.pertussis* BP347 (pDM1)

Lane 6: *B.pertussis* BP347 (pRMB2)



Fresh cultures of *B.pertussis* strains Tohama I, TAB I, BP347, BP347 (pRMB2), BP347 (pDM1) and BP347 (pDM3), H+ and H- (see Section 4.1.5) were set up on BG agar (containing Tc for strains harbouring plasmids). From each culture, a single colony was inoculated into sterile saline. These single colony suspensions were used for two purposes.

i) To determine antibiotic sensitivity of each original colony, for strains Tohama I, TAB I and BP347 an aliquot was spread on BG agar + Tc and on BG agar + Km. For plasmid-carrying strains (known to be Tc resistant) an aliquot was spread on BG agar + Tc + Km. Results are shown in Table 4.6. *B.pertussis* strains Tohama I (the parent strain of BP347) and TAB I (the strain used to construct the genomic library from which clone pRMB2 was isolated) were sensitive to both Tc and Km. Strain BP347 was resistant to Km ( $Km^r$ ), as were BP347 transconjugant strains (transposon Tn5 encodes  $Km^r$ ). Strain BP347 was sensitive to Tc ( $Tc^s$ ) but BP347 transconjugant strains were  $Tc^r$  (plasmid pLAFR1 confers  $Tc^r$ ).

ii) An aliquot from each of the single colony suspensions for *B.pertussis* strains BP347 (pRMB2), BP347 (pDM1), BP347 (pDM3) H+ and BP347 (pDM3) H- was grown to single colonies on BG agar to determine plasmid stability in the absence of tetracycline selection. As controls, an aliquot from each single colony suspension was grown to single colonies on either BG agar (strains Tohama I, TAB I and BP347) or BG agar + Tc (plasmid-carrying strains). After incubation at 35°C for 3 - 4 days, haemolytic activity was assessed for each culture, and the estimated percentage of haemolytic / non-haemolytic colonies is shown in Table 4.6. Antibiotic sensitivities were then determined for representative colonies of each type from each culture plate (see Table 4.6).

Subculture of strains Tohama I, TAB I and BP347 on BG agar had no effect

Table 4.6.

Haemolytic activity and antibiotic sensitivity of BP347 transconjugants in the presence and absence of tetracycline selection

<i>B. pertussis</i> strain	Haemolytic activity <sup>1</sup> and antibiotic sensitivity <sup>2</sup>		
	Original colony	After subculture on BG agar	After subculture on BG agar + Tc
Tohahama I	H <sup>+</sup> , Tc <sup>s</sup> , Km <sup>s</sup>	100%H <sup>+</sup> , Tc <sup>s</sup> , Km <sup>s</sup>	n.t. <sup>3</sup>
TAB I	H <sup>+</sup> , Tc <sup>s</sup> , Km <sup>s</sup>	100%H <sup>+</sup> , Tc <sup>s</sup> , Km <sup>s</sup>	n.t.
BP347	H <sup>-</sup> , Tc <sup>s</sup> , Km <sup>r</sup>	100%H <sup>-</sup> , Tc <sup>s</sup> , Km <sup>r</sup>	n.t.
BP347 (pRMB2)	H <sup>+</sup> , Tc <sup>r</sup> , Km <sup>r</sup>	50%H <sup>+</sup> , Tc <sup>r</sup> , Km <sup>r</sup> 50%H <sup>-</sup> , Tc <sup>s</sup> , Km <sup>r</sup>	100%H <sup>+</sup> , Tc <sup>r</sup> , Km <sup>r</sup>
BP347 (pDM1)	H <sup>+</sup> , Tc <sup>r</sup> , Km <sup>r</sup>	50%H <sup>+</sup> , Tc <sup>r</sup> , Km <sup>r</sup> 50%H <sup>-</sup> , Tc <sup>s</sup> , Km <sup>r</sup>	100%H <sup>+</sup> , Tc <sup>r</sup> , Km <sup>r</sup>
BP347 (pDM3)H <sup>+</sup>	H <sup>+</sup> , Tc <sup>r</sup> , Km <sup>r</sup>	100%H <sup>+</sup> , Tc <sup>s</sup> , Km <sup>s</sup>	100%H <sup>+</sup> , Tc <sup>r</sup> , Km <sup>r</sup>
BP347 (pDM3)H <sup>-</sup>	H <sup>-</sup> , Tc <sup>r</sup> , Km <sup>r</sup>	100%H <sup>-</sup> , Tc <sup>s</sup> , Km <sup>r</sup>	100%H <sup>-</sup> , Tc <sup>r</sup> , Km <sup>r</sup>

approximate

1. The percentages of haemolytic (H<sup>+</sup>) and non-haemolytic (H<sup>-</sup>) colonies are given.
2. s denotes sensitive and r denotes resistance to the antibiotics tetracycline (Tc) and kanamycin (Km).
3. n.t. = not tested.

on haemolytic activity or antibiotic sensitivity. Similarly, when subcultured on BG agar + Tc, haemolytic activity and antibiotic sensitivity of plasmid-carrying strains remained the same as for the original colony in each case.

When strains BP347 (pRMB2) and BP347 (pDM1) were subcultured on BG agar, approximately 50% of colonies were positive and 50% were negative for haemolytic activity. Both types were Km<sup>r</sup>, indicating that they still contained Tn5. Haemolytic colonies were Tc<sup>r</sup>, presumably because these colonies still retained plasmid DNA. However, non-haemolytic colonies were Tc<sup>s</sup>, implying that loss of haemolytic activity accompanied loss of Tc<sup>r</sup> (i.e. plasmid loss).

Strain BP347 (pDM3) H<sup>+</sup> remained haemolytic when subcultured on BG agar. However, colonies were found to be both Tc<sup>s</sup> and Km<sup>s</sup>, implying that in the absence of Tc selection, plasmid (Tc<sup>r</sup>) and transposon Tn5 (Km<sup>r</sup>) had been lost from this strain. These data implied that recombination had occurred between the plasmid-encoded *vir* region of pDM3 and the Tn5-containing *vir* locus of BP347. Strain BP347 (pDM3) H<sup>-</sup> lost Tc<sup>r</sup> (presumably as a result of loss of pDM3 plasmid) but remained Km<sup>r</sup> when subcultured on BG agar.

These results showed that since loss of haemolytic activity was associated with loss of plasmid, the *vir* locus of clones pRMB2 and pDM1 was acting *in-trans* to restore a virulent phenotype to BP347. Plasmid pDM3 acting *in-trans* did not contain sufficient DNA sequence to restore the virulent phenotype to BP347 [BP347 (pDM3) H<sup>-</sup>]. However, where recombination events had occurred between plasmid and chromosomal DNA [BP347 (pDM3) H<sup>+</sup>], the virulent phenotype was restored.

## Section 4.2. RESTRICTION ENZYME MAPPING OF CLONES pDM1 AND pRMB2.

### 4.2.1. Identification of *EcoRI* fragments of pRMB2 showing homology to pDM1.

Digestion of pDM1 with *EcoRI* showed that the genomic insert consisted of fragments E1, E2, E3 and a non-contiguous fragment formed by ligation of the two *EcoRI* to *BamHI* fragments of pRMB2 (Section 4.1.9). Hybridisation analysis was used to determine from which two *EcoRI* fragments of pRMB2, the non-contiguous fragment of pDM1 was derived.

*EcoRI*-digested samples of pRMB2 and pDM1 (and also pDM3 and pLAFR1) were Southern blotted and hybridised with a pDM1 probe (Figure 4.11). The probe hybridised to all of the fragments in the pDM1, pDM3 and pLAFR1 samples. The E1, E2, E3 and pLAFR1 fragments of the probe hybridised to the homologous fragments of pRMB2, and the non-contiguous fragment of the probe hybridised to the E4 and E6 fragments of pRMB2. Therefore the two *EcoRI* to *BamHI* fragments which make up the non-contiguous fragment of pDM1, are contained within the E4 and E6 fragments of pRMB2. The non-contiguous *EcoRI* fragment of pDM1 was designated E4::6.

### 4.2.2. Restriction enzyme analysis of pDM1 and pRMB2 using single and double digests.

In order to define more clearly the regions of clones pDM1 and pRMB2 involved in regulation of expression of virulence-associated factors in *B.pertussis*, a restriction enzyme map was constructed for both clones.

From data accumulated in Section 4.1, restriction enzymes *EcoRI* and *BamHI* appeared to be a logical choice for use in mapping. *EcoRI* was chosen because the genomic insert of pRMB2 was cloned in the *EcoRI* site of pLAFR1, and also the E3 fragment was known to be part of the *vir* locus. *BamHI* was

Figure 4.11.

Southern blot analysis of *EcoRI*-digested plasmid DNA probed with pDM1.

The blot was hybridised with radiolabelled pDM1 plasmid. Numbers on the right refer to the fragment sizes (kb) of *HindIII*-digested  $\lambda$  DNA.

Lane 1: pRMB2

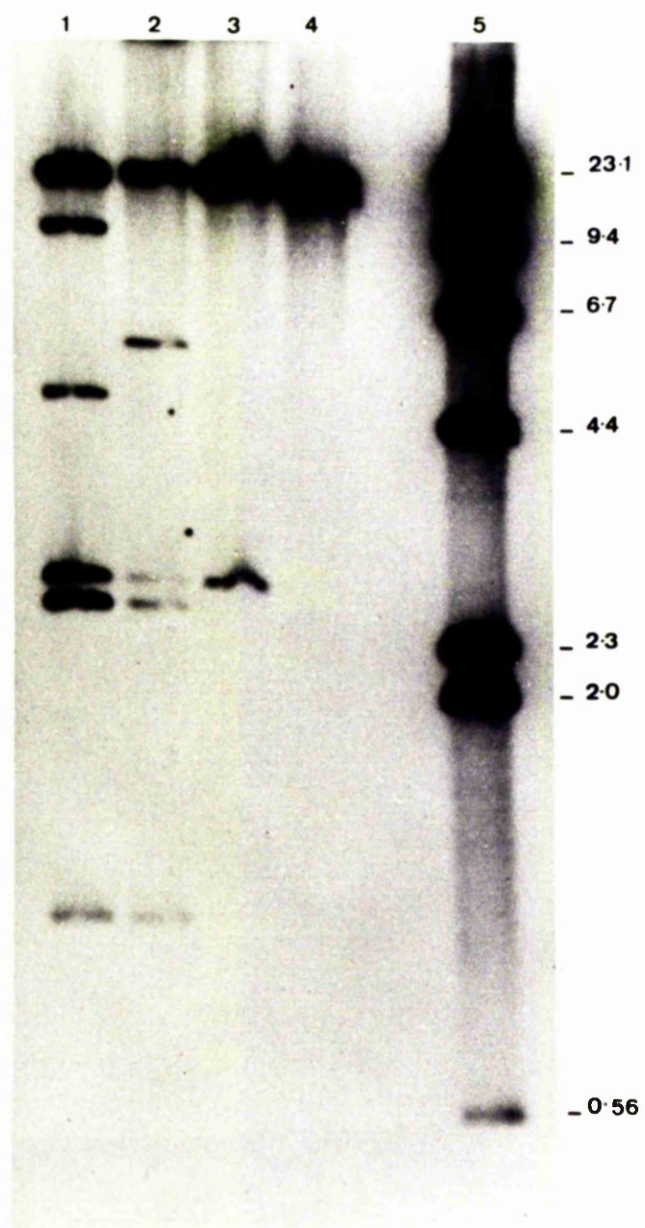
Lane 2: pDM1

Lane 3: pDM3

Lane 4: pLAFR1

Lane 5: <sup>32</sup>P-labelled *HindIII*-digested  $\lambda$  DNA





chosen because this was the enzyme used in construction of pDM1. Since pDM1 contains only one *Bam*HI site, the use of a third enzyme was necessary to obtain a restriction map. Since pLAFR1 contains two *Bgl*III restriction sites, *Bgl*III was the third enzyme chosen for mapping because it would enable orientation of fragments (with respect to pLAFR1 vector) to be determined.

A series of single and double restriction digests using *Eco*RI, *Bam*HI and *Bgl*III was set up for pRMB2 and pDM1 plasmids. Samples were subjected to electrophoresis together with several samples of *Hind*III-digested and *Pst*I-digested  $\lambda$  DNA (Figure 4.12).

Visual inspection of the gel gave an indication of which restriction enzyme site(s) were contained within each fragment. For example, digestion of pDM1 with *Eco*RI gave genomic fragments E1, E2, E3 and E4::6, but when pDM1 was digested with both *Eco*RI and *Bam*HI, the E4::6 fragment was no longer present. This confirmed that E4::6 contained a *Bam*HI site. Similarly, *Eco*RI-digestion of pRMB2 gave genomic fragments E1 to E6. On first inspection, in the sample of pRMB2 digested with both *Eco*RI and *Bam*HI, only the E5 and E6 fragments appeared to be missing. This implied that E5 and E6 both contained one or more *Bam*HI restriction sites. However, E4 must contain at least one *Bam*HI restriction site, since a *Bam*HI to *Eco*RI fragment from E4 is contained within E4::6 (Section 4.2.1). Closer inspection of the gel revealed that the band originally thought to be E4 was in fact slightly larger, and must therefore have arisen as a result of digestion of either E5 or E6 with *Bam*HI. Information about the restriction enzyme site(s) contained within each fragment, deduced from single and double digests data, was therefore corroborated by one of two means, i) single and double restriction digests were hybridised using individual

Figure 4.12.

Electrophoresis of a series of single and double restriction enzyme digests of clones pRMB2 and pDM1.

Ethidium bromide stained gel showing a series of single and double digests of clones pRMB2 and pDM1, using the restriction enzymes *Bam*HI, *Eco*RI and *Bgl*III. Clone pRMB2 digests are shown in (A) and pDM1 digests are shown in (B). Numbers on the left refer to the fragment sizes (kb) of *Hind*III-digested  $\lambda$  DNA. Samples of *Pst*I-digested  $\lambda$  DNA were also included as molecular weight standards. Some of the smaller fragments are barely visible in this photograph, however all fragment sizes are listed in Table 4.7.

H: *Hind*III-digested  $\lambda$  DNA

P: *Pst*I-digested  $\lambda$  DNA

1: *Bam*HI digest

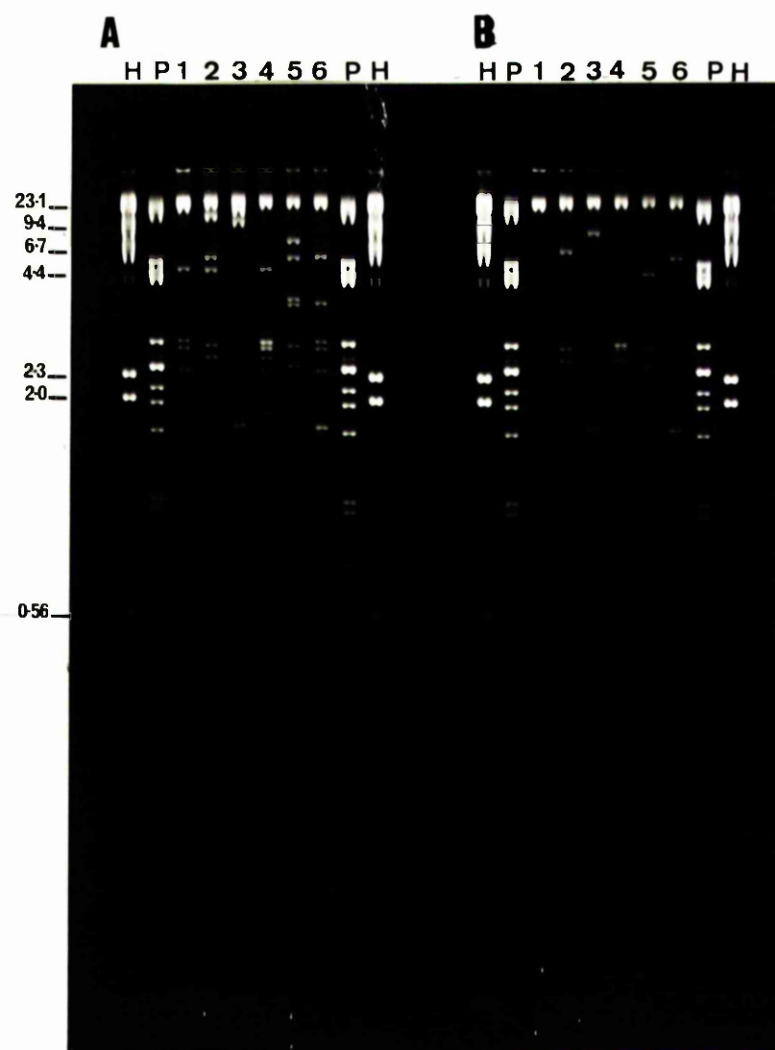
2: *Eco*RI digest

3: *Bgl*III digest

4: *Bam*HI + *Eco*RI digest

5: *Bgl*III + *Eco*RI digest

6: *Bam*HI + *Bgl*III digest



fragments as probes, or ii) individual fragments were isolated and restricted. To obtain a complete picture of the restriction enzyme sites contained within each fragment, the reader is advised to refer to Figure 4.12 whilst analysing data presented below.

To construct restriction maps of pRMB2 and pDM1, it was necessary to determine molecular sizes of the fragments produced by the single and double digest reactions. Band migration distances were measured from the photograph shown in Figure 4.12 by automated densitometry. Using the 11.5kb to 0.514kb fragments of *Pst*I-digested  $\lambda$  DNA as standards, molecular sizes of pRMB2 and pDM1 restriction fragments were estimated (see Table 4.7), by a modification of the method of Plikaytis *et al.* (1986), as described in Section 3.2.7.

This enabled the size of the genomic insert to be determined for each clone, by addition of the sizes of individual *Eco*RI fragments. The E1 to E6 fragments of pRMB2 were estimated to be 1.1kb, 2.5kb, 2.7kb, 4.7kb, 5.1kb and 10.0kb respectively, giving a total insert size of 26.1kb. The E4::6 fragment of pDM1 was estimated as 5.6kb, making a total insert size of 11.9kb for clone pDM1. The sizes of restriction fragments shown in Table 4.7 are used in the analysis of data presented below.

#### 4.2.3. Construction of a restriction enzyme map of pDM1 using hybridisation analysis.

To confirm information deduced from single and double digests data the series of digests of pDM1 was probed with E1, E2, E3 and E4::6, to determine which restriction fragments the individual *Eco*RI fragments hybridised to.

Single and double restriction digests using *Eco*RI, *Bam*HI and *Bgl*III were

Table 4.7.

Estimation of the molecular sizes of fragments produced by a series of single and double restriction enzyme digests of clones pRMB2 and pDM1

Estimated molecular sizes (kb) of restriction fragments <sup>1</sup>						
Clone	<i>Bam</i> HI	<i>Eco</i> RI	<i>Bgl</i> II	<i>Bam</i> HI + <i>Eco</i> RI	<i>Bgl</i> II + <i>Eco</i> RI	<i>Bam</i> HI + <i>Bgl</i> II
pRMB2	* (B6)	*	*	*	*	*
	4.8 (B5)	10.0 (E6)	* (Bg5)	4.8	6.3	5.1
	2.9 (B4)	5.1 (E5)	8.6 (Bg4)	2.8 D	5.1	3.7
	2.7 (B3)	4.7 (E4)	1.7 D	2.7 D	3.7	2.9
	2.4 (B2)	2.7 (E3)	1.6	2.5	3.6	2.7
	1.4 (B1)	2.5 (E2)		2.4	2.7	2.4
		1.1 (E1)		1.9	2.3	1.7 T
				1.4	1.7	1.6
				1.1	1.2	1.4
				1.0	1.1	1.1
					0.55 D	
pDM1	*	*	*	*	*	*
		5.6 (E4::6)	6.8	2.8 D	4.5	5.1
		2.7 (E3)	1.7 D	2.7	2.7	1.7 T
		2.5 (E2)	1.6	2.5	2.3	1.6
		1.1		1.1	1.7	
					1.2	
					1.1	
					0.55 D	

1. The molecular sizes of restriction fragments were estimated from the photograph shown in Figure 4.12 by a modification of the method of Plikaytis *et al.* (1986), as described in Section 3.2.7.

\* indicates a fragment of greater than 10kb

D indicates a doublet band

T indicates a triplet band

( ) indicate designations given to certain fragments

set up for pDM1. Each sample was divided in two and loaded in the wells of two separate gels. After electrophoresis, gels were Southern blotted. One filter was hybridised with oligo-labelled E1 fragment, stripped to remove residual radioactivity, then rehybridised with oligo-labelled E4::6 fragment (Figure 4.13). The second filter was hybridised, first with oligo-labelled E2 fragment, then with oligo-labelled E3 fragment (Figure 4.14).

The E1 probe hybridised to a doublet band of 0.55kb in pDM1 digested with both *BglIII* and *EcoRI*. In *BglIII*-digested pDM1, E1 hybridised to a 1.7kb band. Since E1 contains a *BglIII* site, E1 must have hybridised to a doublet band of 1.7kb. In pDM1 digested with both *BamHI* and *BglIII*, E1 hybridised to the same 1.7kb doublet band, indicating that this region does not contain a *BamHI* restriction site. The information deduced from these data is shown in Figure 4.15a.

The E4::6 probe hybridised to bands of 2.8kb and 5.6kb in pDM1 digested with both *BamHI* and *EcoRI*. However, inspection of the original gel photograph showed that the E4::6 fragment had not been digested to completion by *BamHI*. The E4::6 probe hybridised to bands of 4.5kb and 1.1kb in pDM1 digested with both *BglIII* and *EcoRI*. In pDM1 digested with both *BamHI* and *BglIII*, the E4::6 probe hybridised to bands of 5.1kb and 1.7kb, the latter being a doublet band. These data were collated as shown in Figure 4.15b.

The E2 probe hybridised to a band of 2.3kb in pDM1 digested with both *BglIII* and *EcoRI* (hybridisation to the remaining 0.2kb of E2 was not detected). In *BglIII*-digested pDM1, the E2 probe hybridised to a 6.8kb fragment. In pDM1 digested with both *BamHI* and *BglIII*, the E2 probe hybridised to a band of 5.1kb. These data were assimilated as shown in Figure 4.15c.

Figure 4.13.

Southern blot analysis of a series of single and double restriction digests of pDM1 probed with E1 and E4::6.

The nitrocellulose filter was hybridised with oligo-labelled E1 fragment (A), stripped to remove residual radioactivity, then rehybridised with oligo-labelled E4::6 fragment (B). Numbers on the left refer to the sizes (kb) of the 14.1kb to 1.7kb fragments of *PstI*-digested  $\lambda$  DNA.

Lane 1: *BamHI* digest

Lane 2: *EcoRI* digest

Lane 3: *BglII* digest

Lane 4: *BamHI* + *EcoRI* digest

Lane 5: *BglII* + *EcoRI* digest

Lane 6: *BglII* + *BamHI* digest



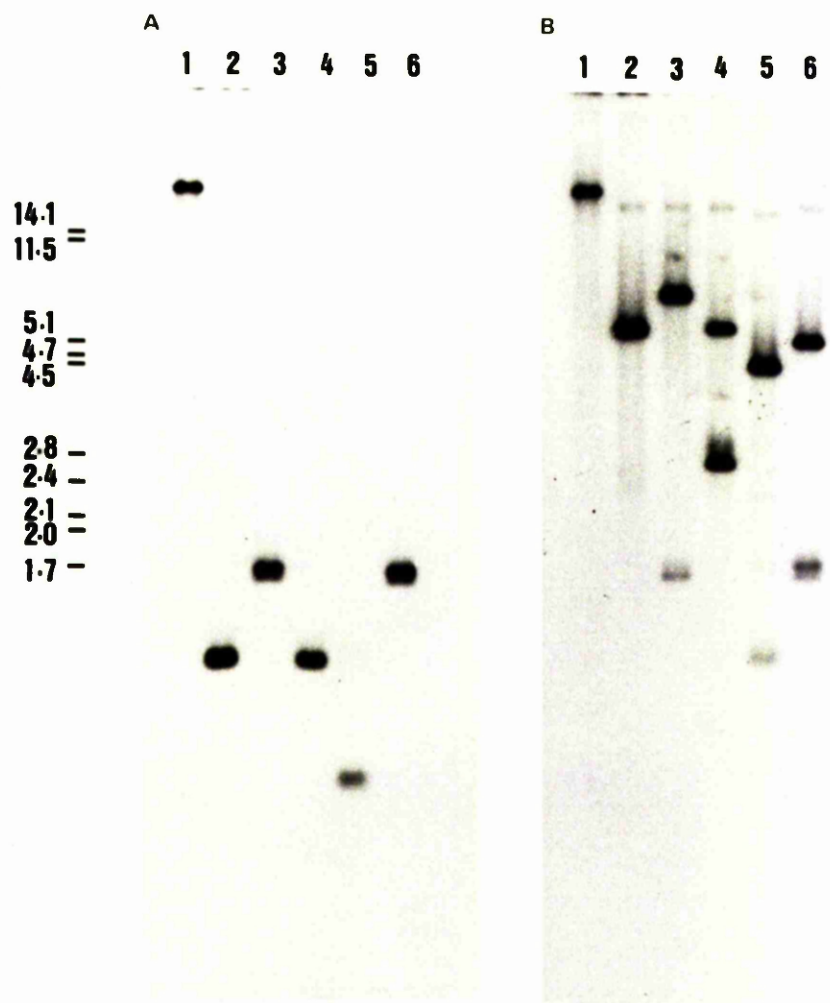


Figure 4.14.

Southern blot analysis of a series of single and double restriction digest:  
of pDM1 probed with E2 and E3.

Probes were prepared by oligo-labelling fragments which had been purified from a gel by centrifugation through a SPIN-X filter unit, and there was minor contamination of the E2 probe with E3 fragment, and vice versa. The nitrocellulose filter was hybridised with the E2 probe (A), stripped to remove residual radioactivity, then rehybridised with the E3 probe (B). Numbers on the left refer to the sizes of the 14.1kb to 1.7kb fragments of *PstI*-digested  $\lambda$  DNA.

Lane 1: *BamHI* digest

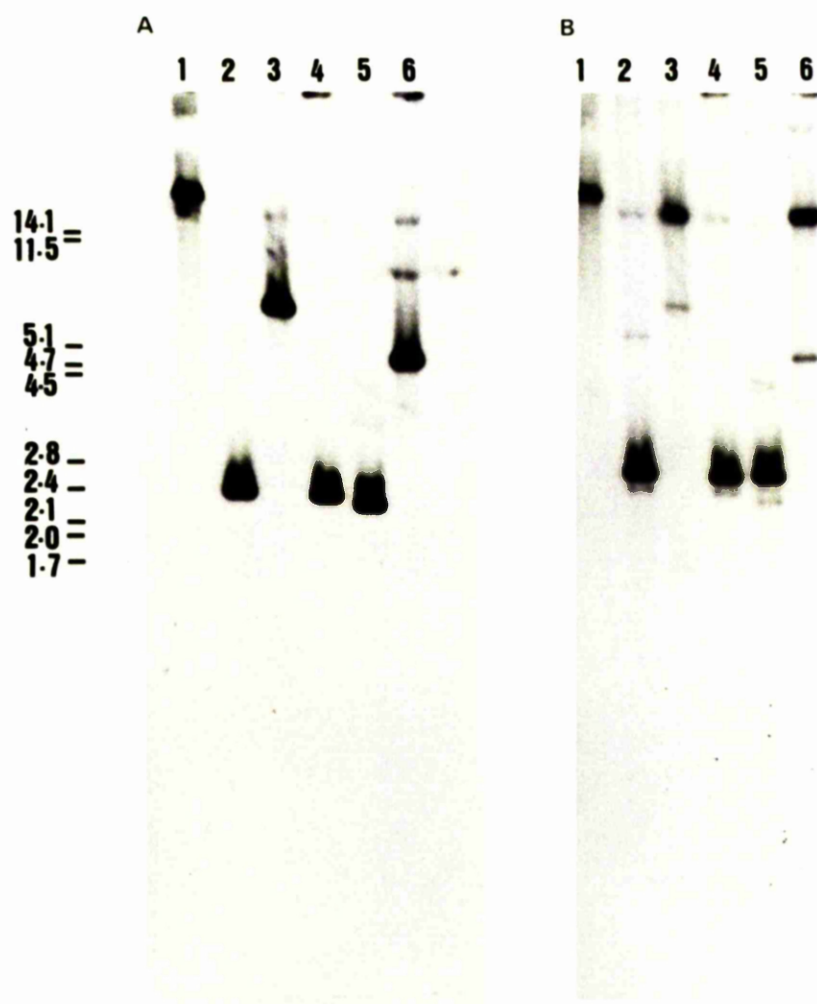
Lane 2: *EcoRI* digest

Lane 3: *BglII* digest

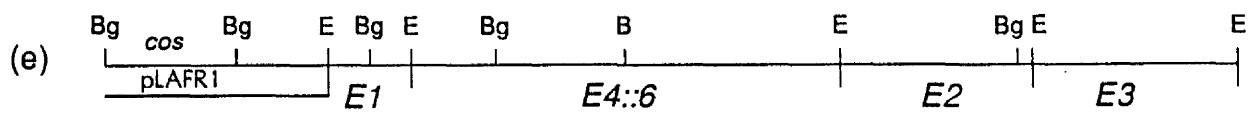
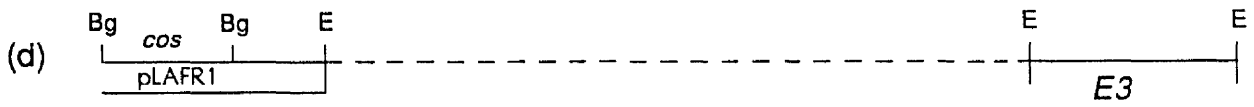
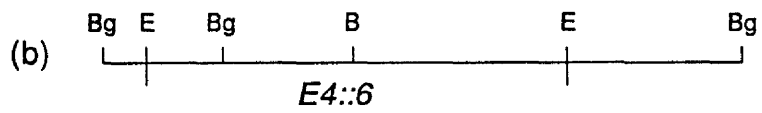
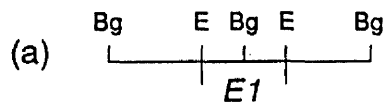
Lane 4: *BamHI* + *EcoRI* digest

Lane 5: *BglII* + *EcoRI* digest

Lane 6: *BamHI* + *BglII* digest



1kb 



The E3 probe hybridised to a large fragment consisting of vector plus genomic DNA in samples of pDM1 digested with *BglIII* and pDM1 digested with both *BamHI* and *BglIII*. This positions E3 at the end of pLAFR1 opposite to that containing the *BglIII* *cos* fragment, as shown in Figure 4.15d.

Compilation of all data enabled a complete restriction enzyme map of pDM1 to be constructed (Figure 4.15e), with regard to *EcoRI*, *BamHI* and *BglIII* sites. The fragment sizes used in construction of the map were those given in Table 4.7. The restriction map shows that the genomic insert of pDM1 consists of two non-contiguous fragments of 3.9kb and 8kb, the 2.7kb *EcoRI* fragment (which harboured the Tn5 insertion in BP347) being contained within the latter. Since pDM1 was able to *trans*-complement BP347, it was deduced that the region essential for regulation of expression of virulence factors in *B.pertussis* is contained within the 8kb portion of pDM1.

#### 4.2.4. Construction of a restriction enzyme map of pRMB2 using data obtained from restriction analysis of individual fragments.

The restriction map of pDM1 was used as a basis for construction of a pRMB2 map. Samples of pRMB2 digested with *EcoRI*, *BamHI* and *BglIII* were subjected to electrophoresis through low melting-point agarose. Fragments E4 to E6, B1 to B5, and the 8.6kb and 12.4kb *BglIII* fragments (Bg4 and Bg5 respectively), were purified from the gel and digested with the restriction enzymes *EcoRI*, *BamHI* and *BglIII*. Samples were subjected to electrophoresis, and the sizes of the restriction fragments were estimated as before (Table 4.8).

Since the E4 fragment contained a 1.1kb *BglIII* to *EcoRI* restriction fragment, it was positioned next to E1 (see Figure 4.15e), enabling the *EcoRI* fragments of pRMB2 to be positioned in the following order, E1, E4,

Table 4.8.

Estimation of the molecular sizes of fragments produced by  
digestion of individual fragments of pRMB2

pRMB2 fragment	Estimated molecular sizes (kb) of restriction fragments <sup>1</sup>		
	<i>EcoRI</i>	<i>BamHI</i>	<i>BglIII</i>
E4	n.t.	1.9, 2.8	1.1, 3.6
E5	n.t.	1.0, 1.4, 2.7	5.1
E6	n.t.	2.4, 2.8, 4.8	3.7, 6.3
B1	1.4	n.t.	1.4
B2	2.4	n.t.	2.4
B3	2.7	n.t.	2.7
B4	1.0, 1.9	n.t.	2.9
B5	4.8	n.t.	1.1, 3.7
Bg4	2.3, 6.3	1.1, 2.4, 5.1	n.t.
Bg5	3.6, 3.7, 5.1	1.4, 1.7, 2.7	n.t.
		2.9, 3.7	

1. Individual fragments of pRMB2 were digested with the restriction enzymes *EcoRI*, *BamHI* and *BglIII*. Samples were subjected to electrophoresis, and the sizes of fragments produced were estimated from a photograph of the ethidium bromide stained gel by a modification of the method of Plikaytis et al. (1986), as described in Section 3.2.7.

n.t. = not tested.

E5, E6, E2, E3.

A point worthy of note is that fragments E4, E5 and E6 contained one, two and two *Bam*HI restriction sites respectively, but *Bam*HI-digestion of pRMB2 produced six fragments. This implied that a *Bam*HI site and *Eco*RI site were located very close together in pRMB2.

Assimilation of all restriction digest data enabled the construction of a restriction enzyme map of pRMB2 (Figure 4.16). Because the 1.4kb and 2.7kb *Bam*HI fragments (B1 and B3 respectively) did not contain either *Eco*RI or *Bgl*II restriction sites, their orientation could not be determined from available data.

#### 4.2.5. Comparison of the restriction map of pRMB2 with a recently published map of a region containing the *B.pertussis vir* and *fha* loci.

Stibitz *et al.* (1988a) published a restriction map of a clone (pUW21-26) which contained a region of the *B.pertussis* chromosome encompassing the *vir* and *fha* loci. Clone pUW21-26 was isolated from a gene library of *B.pertussis* BP338 (a derivative of strain Tohama I) constructed by cloning genomic DNA which had been partially digested with *Sau*3A into cosmid vector pHC79 (Stibitz *et al.*, 1988a). Comparison of the restriction map of pRMB2 with that of pUW21-26 shows homology for a 15.2kb region (E3, E2 and E6; Figure 4.16), but the remaining 10.9kb of pRMB2 (E5, E4 and E1; Figure 4.16) has a different restriction pattern.

The *vir* locus described by Stibitz *et al.* (1988) is contained within a large *Cla*I fragment of approximately 22kb. Therefore, pRMB2 DNA was analysed to determine if it contained a homologous *Cla*I fragment. Restriction analysis showed that pRMB2 plasmid DNA was not digested by either *Cla*I or the isoschizomer *Bsc*I.

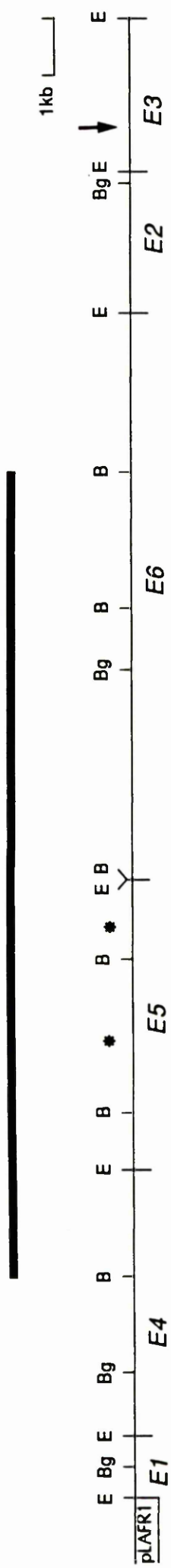
Figure 4.16.

Restriction map of pRMB2.

The bar above represents the region deleted after digestion of the cosmid with *Bam*HI and religation to form pDM1. Designations E1 to E6 represent *Eco*RI fragments in order of increasing size. The arrow indicates the site of Tn5 insertion in BP347. The region essential for regulation of virulence factors in *B.pertussis* is contained within an 8kb region, as indicated by the bar below. Asterisks denote that the order of the 1.4kb and 2.7kb *Bam*HI fragments was not determined.

E, *Eco*RI; B, *Bam*HI; Bg, *Bgl*III.





*ClaI*-digested genomic DNA from *B.pertussis* strains L84 I, BP347, Tohama I and TAB I was Southern blotted and hybridised using the E3 fragment of pRMB2 (which is part of the *vir* locus) as a probe (Figure 4.17). The E3 probe hybridised to the fragment of approximately 22kb in all samples, except BP347 which contained Tn5 (there are no *ClaI* restriction sites in transposon Tn5). There was no apparent difference in this region between the *B.pertussis* strains which were used to construct the genomic libraries from which clones pRMB2 and pUW21-26 were isolated i.e. strains TAB I and Tohama I respectively.

The gene library from which pRMB2 was isolated was constructed by cloning *B.pertussis* TAB I DNA which had been partially digested with *EcoRI* into cosmid vector pLAFR1 (Brownlie *et al.*, 1986). It was therefore possible that the genomic insert of pRMB2 was formed by ligation of two non-contiguous genomic fragments. To test this theory, *BglIII*-digested DNA from plasmid pRMB2 and from *B.pertussis* strains L84 I, BP347, Tohama I and TAB I was Southern blotted and hybridised with a probe made from the 12.4kb *BglIII* (Bg5) fragment of pRMB2 (Figure 4.18). The Bg5 fragment was chosen as a probe because it spans the junction between the E5 and E6 fragments. The probe hybridised to the Bg5 fragment in the pRMB2 sample. However in genomic DNA samples, the Bg5 probe hybridised to two bands. This data supported the theory that the genomic insert of pRMB2 consists of two non-contiguous fragments, of 15.2kb (E3, E2 and E6) and 10.9kb (E5, E4 and E1). The 15.2kb fragment shows close homology to the restriction map of the *vir* locus published by Stibitz *et al.* (1988a).

Figure 4.17.

Southern blot analysis of *ClaI*-digested *B.pertussis* DNA probed with E3.

The nitrocellulose filter was hybridised with oligo-labelled E3 fragment.

Numbers on the left refer to fragment sizes (kb) of *HindIII*-digested  $\lambda$  DNA.

32  
Lane 1: P-labelled *HindIII*-digested  $\lambda$  DNA  
Lane 2: *B.pertussis* L84 I  
Lane 3: *B.pertussis* BP347  
Lane 4: *B.pertussis* Tohama I  
Lane 5: *B.pertussis* TAB I

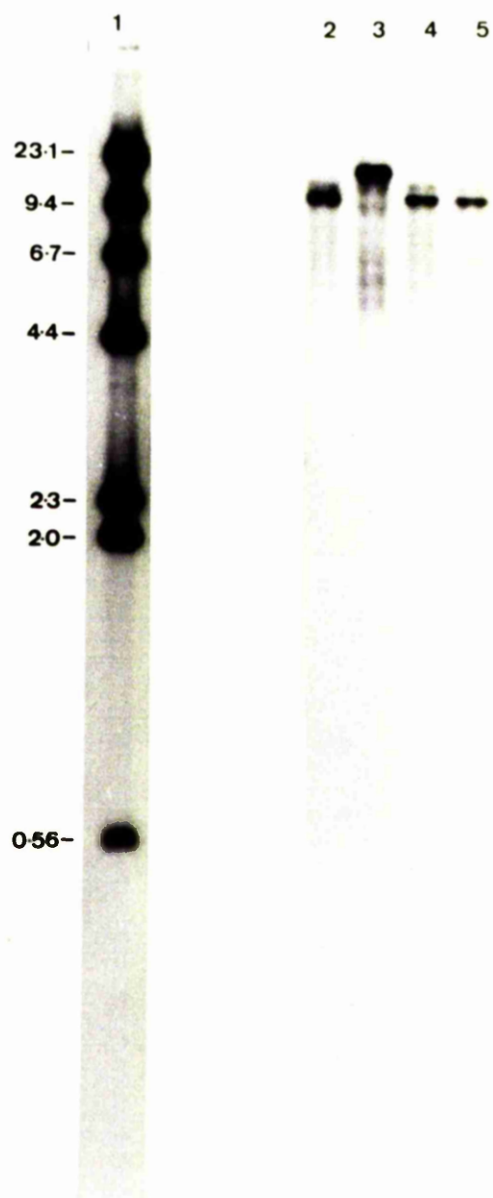


Figure 4.18.

Southern blot analysis of *BglII*-digested *B.pertussis* DNA probed with Bg5.

The blot was hybridised using a probe made by oligo-labelling Bg5 fragment which had been purified from a gel by centrifugation through a SPIN-X filter unit. (There was minor contamination of the probe with pLAFR1 vector and the Bg4 fragment.) The position of the Bg5 fragment is marked by the arrow to the right. The two arrows on the left mark the fragments to which Bg5 hybridised in genomic DNA samples. Numbers on the left refer to the sizes (kb) of *HindIII*-digested  $\lambda$  DNA molecular weight standards.

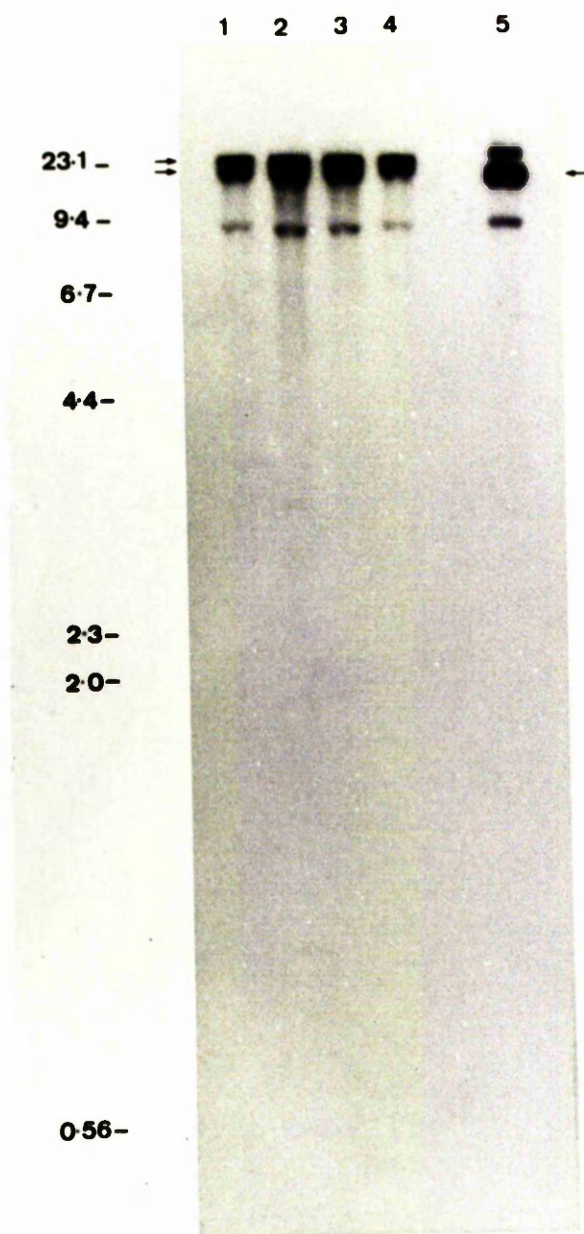
Lane 1: *B.pertussis* L84 I

Lane 2: *B.pertussis* BP347

Lane 3: *B.pertussis* Tohama I

Lane 4: *B.pertussis* TAB I

Lane 5: pRMB2



Section 4.3. MOLECULAR ANALYSIS OF THE VIR REGION IN THE OTHER BORDETELLA SPECIES.

4.3.1. Identification of regions homologous to *vir* in the other *Bordetella* species.

Many of the virulence determinants produced by *B.pertussis*, such as AC, HLY and FHA, are also produced by virulent strains of *B.parapertussis* and *B.bronchiseptica*. Production of some virulence determinants e.g. HLT, is common to all four species of the genus. Since expression of virulence-associated factors in *B.pertussis* is regulated by the *vir* locus, it was of interest to determine if the other species in the genus contained an homologous region.

The functional *vir* locus of *B.pertussis* contained at least the E3 and E2 fragments, therefore *EcoRI* was chosen for preliminary hybridisation analysis. *EcoRI*-digested DNA from several strains of all species of *Bordetella* was subjected to electrophoresis, together with *EcoRI*-digested pRMB2 DNA. The gel was Southern blotted and hybridised with a pRMB2 probe (Figure 4.19).

Strains of *B.pertussis*, *B.parapertussis* and *B.bronchiseptica* showed a similar pattern of hybridisation. Apart from BP347 (Vir-) where E3 was replaced by the larger Tn5-containing fragment, *B.pertussis* strain 11615, an avirulent phase variant, seemed to lack E2 and instead exhibited a fragment of higher molecular weight which hybridised to the probe. However there were no detectable differences between *EcoRI*-digested DNA from virulent (phase I) and avirulent (phase III and phase IV) strains of *B.pertussis* L84 and *B.bronchiseptica* FW5. Similarly there was no detectable difference between *EcoRI*-digested DNA from *B.pertussis* strains 7R and 7R(34), the latter strain being an avirulent derivative of strain 7R,

Figure 4.19.

Southern blot analysis of *EcoRI*-digested *Bordetella* DNA probed with pRMB2.

The blot was hybridised with oligo-labelled pRMB2 plasmid. E1 to E6 and pLAFR1 (molecular sizes of 1.1kb, 2.5kb, 2.7kb, 4.7kb, 5.1kb, 10.0kb and 21.6kb respectively) indicate the fragments observed in pRMB2. The arrow shows the position of a fragment in *B.avium* samples which hybridised faintly to pRMB2 (more clearly seen in Figure 4.22), but strongly to pDM30.

Lane 1: pRMB2

Lane 2: *B.pertussis* L84 I

Lane 3: *B.pertussis* L84 IV

Lane 4: *B.pertussis* BP347

Lane 5: *B.pertussis* 11615

Lane 6: *B.pertussis* 44122/7R

Lane 7: *B.pertussis* 44122/7R(34)

Lane 8: *B.parapertussis* 59521

Lane 9: *B.parapertussis* 10520

Lane 10: *B.bronchiseptica* FW5 I

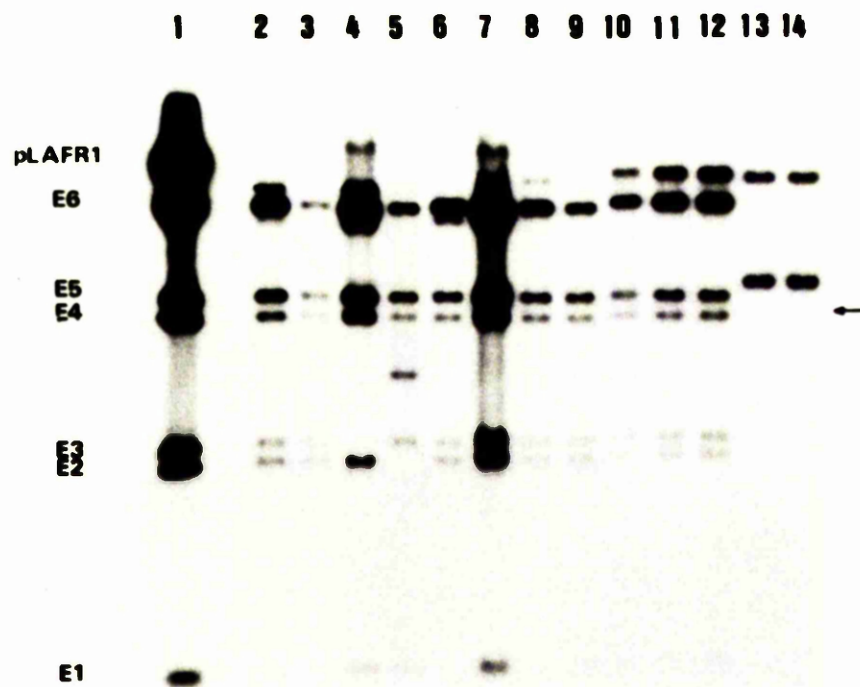
Lane 11: *B.bronchiseptica* FW5 III

Lane 12: *B.bronchiseptica* 276 I

Lane 13: *B.avium* 4091

Lane 14: *B.avium* 4148





obtained by repeated subculture. As well as fragments E1 to E6, all *B.bronchiseptica* samples exhibited an additional band of higher molecular weight when probed with pRMB2. A faint additional band was also exhibited in several of the *B.pertussis* and *B.parapertussis* samples. (The size of the additional higher molecular weight band was different for each of the three species). DNA from *B.avium* strains exhibited a markedly different hybridisation pattern to the other *Bordetella* species.

The filter was stripped to remove residual radioactivity, and rehybridised using the more specific E3 fragment as a probe (Figure 4.20). The E3 fragment hybridised to similar sized fragments in all samples except BP347, which contained Tn5, and the two *B.avium* samples. The band which hybridised in the *B.avium* samples with the E3 probe was present only as a faint band when samples were hybridised with pRMB2. In the *B.pertussis* 7R(34) sample (which contained an excess of DNA compared to all other samples) the E3 probe also hybridised to the E5 fragment. The band exhibited in *B.bronchiseptica* samples appeared to be marginally larger than the equivalent band in samples of *B.pertussis* and *B.parapertussis* (this was also observed when the filter was hybridised with the pRMB2 probe).

The filter was stripped to remove residual radioactivity and rehybridised using a pLAFR1 plasmid probe (results not shown). The probe hybridised to the pLAFR1 band of *EcoRI*-digested pRMB2 DNA. There was no hybridisation of pLAFR1 to the genomic DNA samples.

To gain further information on the regions homologous to *vir* in the other *Bordetella* species, a second enzyme (*Bam*HI) was used for hybridisation analysis. *Bam*HI-digested DNA from pRMB2 and from strains of all *Bordetella* species was subjected to electrophoresis in two separate gels, and Southern blotted. One filter was hybridised with a pRMB2 probe

Figure 4.20.

Southern blot analysis of *EcoRI*-digested *Bordetella* DNA probed with pDM30.

The blot shown in Figure 4.19 was stripped to remove residual radioactivity and rehybridised with a pDM30 probe. For reference, the positions of the E1 to E6 and pLAFR1 fragments of pRMB2 are also shown in this Figure (A).

Lane 1: pRMB2

Lane 2: *B.pertussis* L84 I

Lane 3: *B.pertussis* L84 IV

Lane 4: *B.pertussis* BP347

Lane 5: *B.pertussis* 11615

Lane 6: *B.pertussis* 44122/7R

Lane 7: *B.pertussis* 44122/7R(34)

Lane 8: *B.parapertussis* 59521

Lane 9: *B.parapertussis* 10520

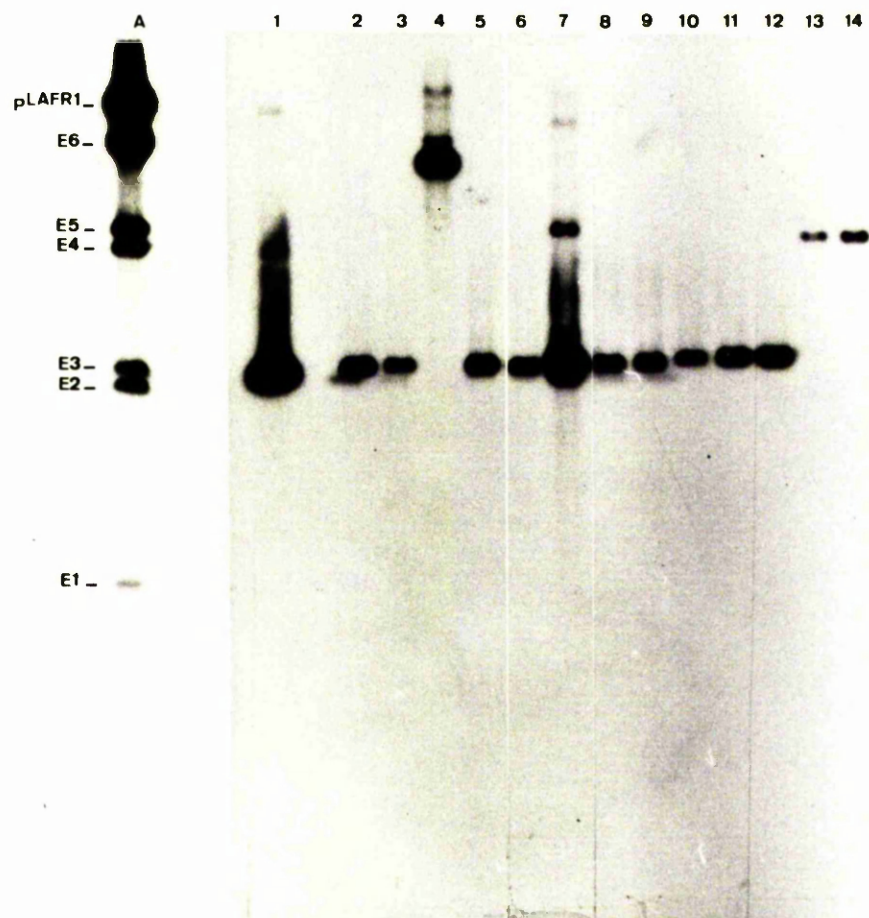
Lane 10: *B.bronchiseptica* FW5 I

Lane 11: *B.bronchiseptica* FW5 III

Lane 12: *B.bronchiseptica* 276 I

Lane 13: *B.avium* 4091

Lane 14: *B.avium* 4148



and the other was hybridised with the more specific E3 probe (Figure 4.21).

Genomic DNA samples from strains of *B.pertussis*, *B.parapertussis* and *B.bronchiseptica* hybridised to fragments B1 to B5 of pRMB2. The *Bam*HI to *Eco*RI terminal portions of the genomic insert of pRMB2 (which together with pLAFR1 comprise B6) each hybridised to a large *Bam*HI fragment of genomic DNA in samples from strains of *B.pertussis*, *B.parapertussis* and *B.bronchiseptica*. However the fragments exhibited in strains of *B.pertussis* were approximately 12kb and 14kb, but those exhibited in strains of *B.parapertussis* and *B.bronchiseptica* were approximately 6.5kb and 16kb. (The molecular sizes of fragments were estimated from a graph of migration distance against log DNA length, plotted for *Hind*III-digested  $\lambda$  DNA molecular weight standards). The pRMB2 probe also hybridised to an additional band in DNA samples from *B.pertussis*, *B.parapertussis* and *B.bronchiseptica* strains. This band differed in molecular size for the three species, being approximately 4.2kb, 6.4kb and 7.5kb for strains of *B.pertussis*, *B.parapertussis* and *B.bronchiseptica* respectively. The pRMB2 probe hybridised to only one band (of approximately 6.7kb) in the *B.avium* sample.

The E3 probe (which is part of the *vir* locus) hybridised to the 14kb band in *B.pertussis* DNA samples, and to the 16kb band in *B.parapertussis* and *B.bronchiseptica* samples. No hybridisation to the E3 probe was detected in the *B.avium* sample.

#### 4.3.2. Further analysis of the region homologous to *vir* in *B.avium* strains.

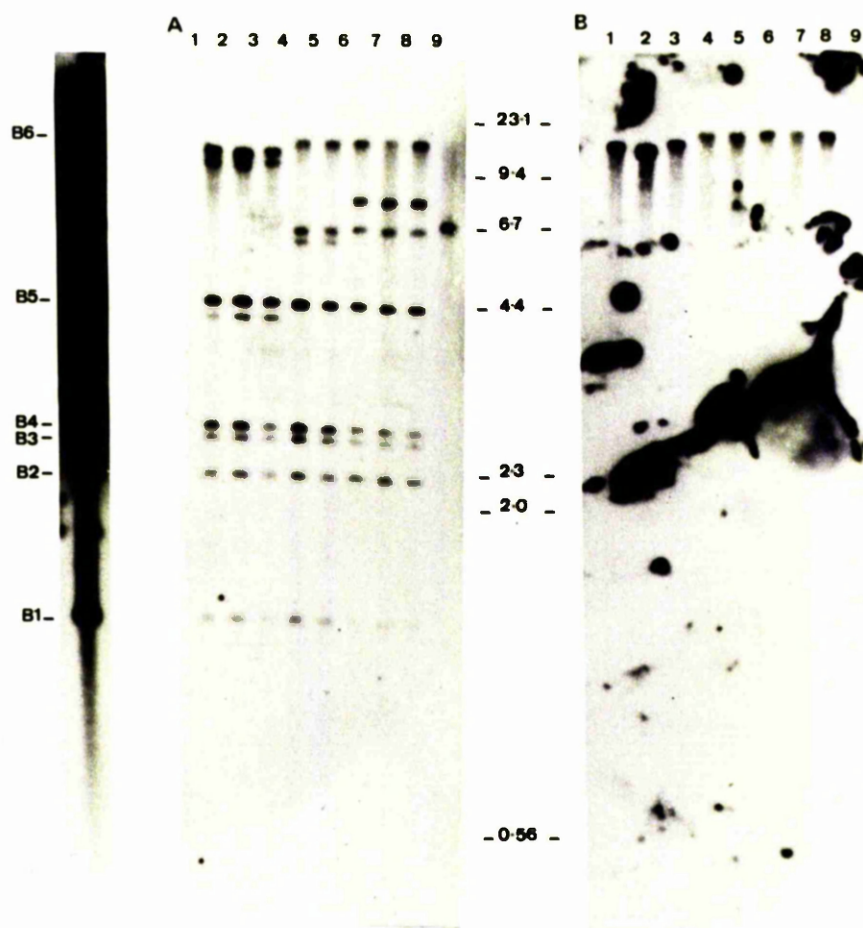
Since the region showing homology to *vir* in DNA from *B.avium* strains 4091 and 4148 was markedly different to that of the other *Bordetella* species, hybridisation analysis was extended to cover a larger number of

Figure 4.21.

Southern blot analysis of *Bam*HI-digested *Bordetella* DNA probed with pRMB2 and pDM30.

The nitrocellulose filters were probed with pRMB2 (A) and pDM30 (B). Numbers between (A) and (B) refer to fragment sizes (kb) of *Hind*III-digested  $\lambda$  DNA. B1 to B6 denote the fragments observed in *Bam*HI-digested pRMB2 DNA.

Lane 1: *B.pertussis* L84 I  
Lane 2: *B.pertussis* Tohama I  
Lane 3: *B.pertussis* TAB I  
Lane 4: *B.parapertussis* 59521  
Lane 5: *B.parapertussis* 10520  
Lane 6: *B.bronchiseptica* FW5 I  
Lane 7: *B.bronchiseptica* FW5 III  
Lane 8: *B.bronchiseptica* 276 I  
Lane 9: *B.avium* 4091



*B.avium* strains. DNA from several *B.avium* strains, and from two *B.pertussis* strains (for comparison) was digested with *EcoRI*. Samples were subjected to electrophoresis together with *EcoRI*-digested pRMB2 DNA. The gel was Southern blotted onto Hybond N nylon membrane (Amersham) and hybridised with a pRMB2 probe (Figure 4.22A).

The pattern of hybridisation exhibited by *B.avium* strains was markedly different from that of the *B.pertussis* strains. Hybridisation of pRMB2 to *B.pertussis* strains has been reported previously (e.g. Section 4.3.1). Bands, of approximately 5.3kb and 17kb, and a faint band of 4.9kb were present in DNA samples from *B.avium* strains GOBL118, GOBL124, GOBL136, GOBL141, GOBL142A, 4091 and 4148. Two bands of approximately 5.2kb and 17kb were present in DNA samples from *B.avium* strains GOBL110 and GOBL122. Additional bands were also present in some of the *B.avium* samples.

The filter was stripped to remove residual radioactivity and rehybridised with a pLAFR1 plasmid probe (Figure 4.22B). The pLAFR1 probe hybridised to the pLAFR1 fragment in *EcoRI*-digested pRMB2 DNA, and to the additional bands which were present in *B.avium* samples when probed with pRMB2.

Residual radioactivity was removed, and the filter was rehybridised with the more specific E3 fragment of pRMB2 as a probe (Figure 4.23). In *B.pertussis* samples, E3 hybridised to a 2.7kb fragment (strain L84 I) and to the Tn5-containing 8.4kb fragment (strain BP347). In samples from *B.avium* strains GOBL118, GOBL124, GOBL136, GOBL141, GOBL142A, 4091 and 4148, the E3 probe hybridised to the 4.9kb band (this band was present only faintly when samples were hybridised with the pRMB2 probe). However in *B.avium* strains GOBL110 and GOBL122, the E3 probe hybridised to the 5.2kb fragment.



Figure 4.22.

Southern blot analysis of *EcoRI*-digested *B.avium* DNA probed with pRMB2 and pLAFR1.

Southern blot hybridisation analysis was performed as described in Section 3.2.9, using Hybond-N nylon membrane (Amersham). The filter was hybridised with a pRMB2 probe (A), stripped to remove residual radioactivity, and rehybridised with a pLAFR1 probe (B). The numbers between (A) and (B) refer to fragment sizes (kb) of *HindIII*-digested  $\lambda$  DNA. The bars to the left of (A) mark the positions of the E1 to E6 and pLAFR1 fragments of pRMB2.

32  
Lane 1: P-labelled *HindIII*-digested  $\lambda$  DNA

Lane 2: pRMB2

Lane 3: *B.pertussis* L84 I

Lane 4: *B.pertussis* BP347

Lane 5: *B.avium* GOBL118

Lane 6: *B.avium* GOBL124

Lane 7: *B.avium* GOBL136

Lane 8: *B.avium* GOBL141

Lane 9: *B.avium* GOBL142A

Lane 10: *B.avium* GOBL110

Lane 11: *B.avium* GOBL122

Lane 12: *B.avium* 4091

Lane 13: *B.avium* 4148

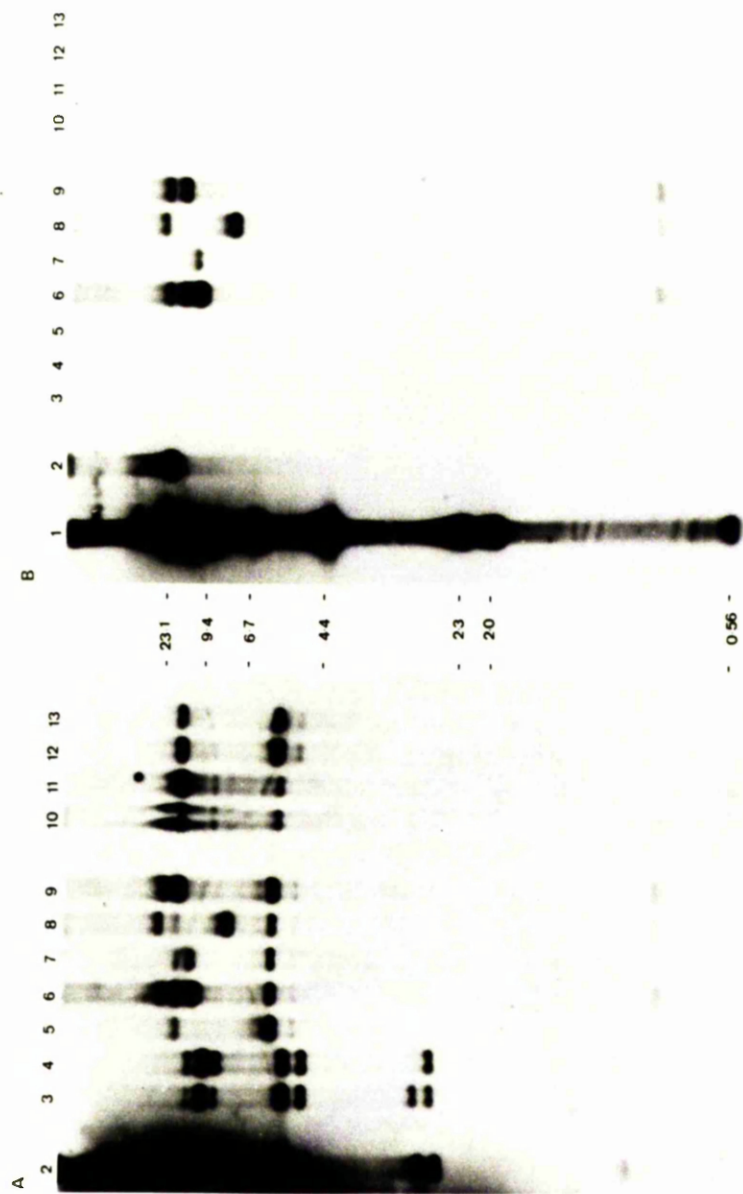
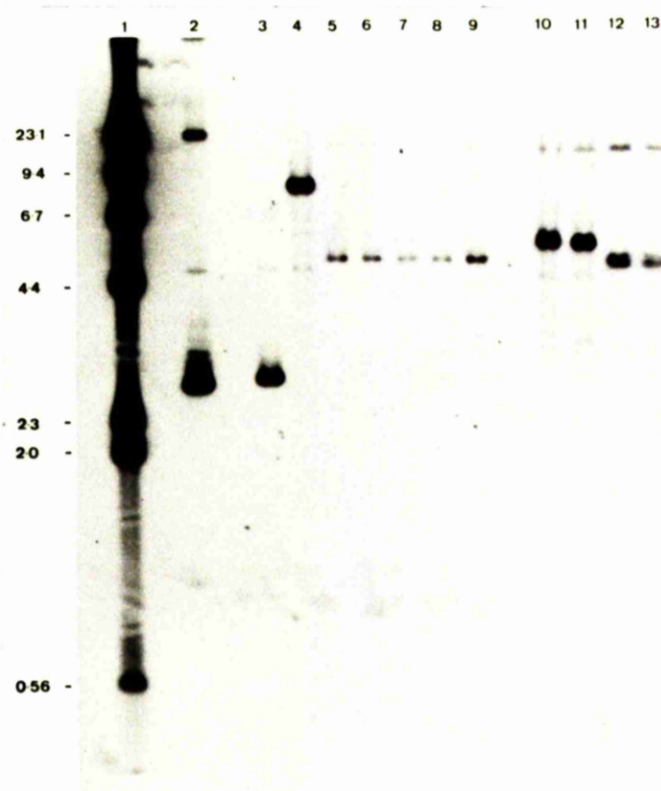


Figure 4.23.

Southern blot analysis of *EcoRI*-digested *B.avium* DNA probed with E3.

The blot shown in Figure 4.22 was stripped to remove residual radioactivity, and rehybridised with oligo-labelled E3 fragment. The probe may have been slightly contaminated with other pRMB2 fragments, which possibly accounts for faint hybridisation to the E4 fragment in *B.pertussis* samples, and the 17kb fragment in *B.avium* samples. Numbers on the left refer to fragment sizes (kb) of *HindIII*-digested  $\lambda$  DNA.

32  
Lane 1: P-labelled *HindIII*-digested  $\lambda$  DNA  
Lane 2: pRMB2  
Lane 3: *B.pertussis* L84 I  
Lane 4: *B.pertussis* BP347  
Lane 5: *B.avium* GOBL118  
Lane 6: *B.avium* GOBL124  
Lane 7: *B.avium* GOBL136  
Lane 8: *B.avium* GOBL141  
Lane 9: *B.avium* GOBL142A  
Lane 10: *B.avium* GOBL110  
Lane 11: *B.avium* GOBL122  
Lane 12: *B.avium* 4091  
Lane 13: *B.avium* 4148



Hybridisation analysis showed that although structurally well conserved within the species, the region in *B.avium* samples which showed homology to the pRMB2 and E3 probes was markedly different from the other species of *Bordetella*.

#### Section 4.4. COMPLEMENTATION OF PHASE VARIANT *BORDETELLA* STRAINS BY pDM1.

##### 4.4.1. Complementation of *B.pertussis* L84 IV by pDM1.

Expression of virulence determinants in *B.pertussis* is affected by a process called phase variation, a genotypic change in which avirulent variants arise in the population upon repeated subculture *in vitro*. Brownlie *et al.* (1988) showed that clone pRMB2 restored expression of virulence factors to *B.pertussis* L84 IV, and it was of interest to determine if the *vir* locus contained within pDM1 could also *trans*-complement the strain.

Clone pDM1 was transferred by conjugation from *E.coli* JM83 to *B.pertussis* L84 IV. Donor strain *E.coli* HB101 (pLAFR1) was included as a negative control. Conjugation mixes were incubated at 35°C for 8h. Cells were then transferred to selective BG agar. As controls, donor, helper and recipient strains were spread on selective BG agar. After incubation at 35°C for 3 - 4 days, the *B.pertussis* L84 IV (pLAFR1) plate supported growth of several hundred non-haemolytic colonies. The *B.pertussis* L84 IV (pDM1) plate supported growth of 60 colonies, only 10% of which were haemolytic. The morphology of cells was checked by Gram staining and found to be like that of *B.pertussis*. Recipient strain L84 IV used as a control was able to grow on selective BG agar, therefore the non-haemolytic colonies on the

*B.pertussis* L84 IV (pDM1) plate (which were also negative for AC and FHA activities) probably did not contain plasmid. The haemolytic colonies were presumed to contain pDM1 plasmid.

To determine if expression of other virulence-associated factors had been restored, *B.pertussis* L84 IV (pDM1) was assayed for AC and FHA activities. *B.pertussis* L84 I was included as a positive control, and *B.pertussis* strains L84 IV and L84 IV (pLAFR1) were included as negative controls. Results are shown in Table 4.9. Clone pDM1 (like pRMB2) restored expression of virulence determinants to *B.pertussis* L84 IV.

#### 4.4.2. Complementation of phase III *B.bronchiseptica* strains by pDM1.

Hybridisation studies (Section 4.3.1) showed that *B.bronchiseptica* contained a region of DNA homologous to the *vir* locus of *B.pertussis* (however this region had a slightly different *sequence* for the two species). Presumably the *vir* locus of *B.bronchiseptica* controls expression of virulent phase genes, in a manner similar to that of *B.pertussis*. Clone pDM1 was transferred to several phase III *B.bronchiseptica* strains to determine if the *vir* locus of *B.pertussis* could *trans*-complement the phase III *B.bronchiseptica* strains.

Using a tri-parental mating system which included the use of a helper plasmid (pRK2013), clone pDM1 was transferred by conjugation from *E.coli* JM83 to *B.bronchiseptica* strains AS1 III, FW5 III and 276 III. Donor culture *E.coli* HB101 (pLAFR1) was also included as a negative control. Conjugation mixes were incubated at 35°C for 8h. Cells were then transferred to selective BG agar. Donor, helper and recipient strains used as controls were unable to grow on selective BG agar. After incubation at 35°C for 2 days, plates supported growth of several hundred colonies.

Table 4.9.

Complementation of *B. pertussis* L84 IV by pDM1

<i>B. pertussis</i> strain	Phenotype <sup>a</sup>		
	HLV	AC	FHA
L84 I	+	+	+
L84 IV	-	-	-
L84 IV (pLAFR1)	-	-	-
L84 IV (pDM1)	+	+	+

a. HLV = haemolysin, AC = adenylate cyclase, FHA = filamentous haemagglutinin.

Transconjugant strains AS1 III (pLAFR1), FW5 III (pLAFR1) and 276 III (pLAFR1) possessed a colonial morphology characteristic of phase III *B.bronchiseptica* i.e. non-haemolytic, large, flat colonies. However, transconjugant strains AS1 III (pDM1), FW5 III (pDM1) and 276 III (pDM1) possessed a small, domed colonial morphology characteristic of phase I *B.bronchiseptica*. The percentage of transconjugant colonies showing haemolysis varied for each of the three *B.bronchiseptica* strains, being 100% for AS1 III (pDM1), approximately 50% for FW5 III (pDM1) and approximately 10% for 276 III (pDM1). When the conjugation experiment was repeated, transconjugant *B.bronchiseptica* strains AS1 III (pDM1), FW5 III (pDM1) and 276 III (pDM1) again possessed a small, domed colonial morphology, but the percentage of haemolytic colonies ranged from approximately 5% to 20% for the three strains. When single haemolytic colonies of *B.bronchiseptica* AS1 III (pDM1), FW5 III (pDM1) and 276 III (pDM1) were subcultured to single colonies on BG agar + Tc, haemolytic activity could only be detected in approximately 75% of the colonies produced for each strain.

Single colonies from *B.bronchiseptica* AS1 III (pLAFR1), FW5 III (pLAFR1), 276 III (pLAFR1) and AS1 III (pDM1) transconjugant plates, two haemolytic colonies from the FW5 III (pDM1) transconjugant plate, and two haemolytic plus two non-haemolytic colonies from the 276 III (pDM1) transconjugant plate were grown for the purpose of assay for adenylate cyclase. Included in the assay were *B.bronchiseptica* strains AS1 I, FW5 I and 276 I as positive controls, and strains AS1 III, FW5 III and 276 III as negative controls. *B.pertussis* strains L84 I and BP347 were included as additional positive and negative controls respectively. Production of AC was assayed by the development of a blue colour from X-gal in the wells of



a microtitre tray. Samples were set up in duplicate, and results are shown in Figure 4.24. *B.bronchiseptica* phase I strains were positive, and phase III strains were negative for AC activity. *B.bronchiseptica* transconjugant strains AS1 III (pLAFR1), FW5 III (pLAFR1) and 276 III (pLAFR1) were negative for AC activity. All *B.bronchiseptica* phase III transconjugant samples containing pDM1 were positive for AC activity. Surprisingly, samples which were grown from the two non-haemolytic *B.bronchiseptica* 276 III (pDM1) transconjugant colonies proved positive for AC activity.

Single haemolytic pDM1 transconjugant colonies, and single non-haemolytic pLAFR1 transconjugant colonies of *B.bronchiseptica* strains AS1 III, FW5 III and 276 III were grown for the purpose of FHA assay. Included in the assays were *B.bronchiseptica* strains AS1 III, FW5 III and 276 III as negative controls, and *B.pertussis* strains L84 I and BP347 as positive and negative controls respectively. Results for *B.bronchiseptica* AS1 are shown in Figure 4.25. No haemagglutination was observed in *B.bronchiseptica* AS1 III and AS1 III (pLAFR1) samples. Haemagglutination was detected in *B.bronchiseptica* AS1 (pDM1) samples. It would have been of interest to compare the haemagglutination titre of *B.bronchiseptica* strain AS1 III (pDM1) with that of strain AS1 I, regrettably the latter was not included in the assay. FHA assays for *B.bronchiseptica* FW5 and *B.bronchiseptica* 276 gave similar results to *B.bronchiseptica* AS1.

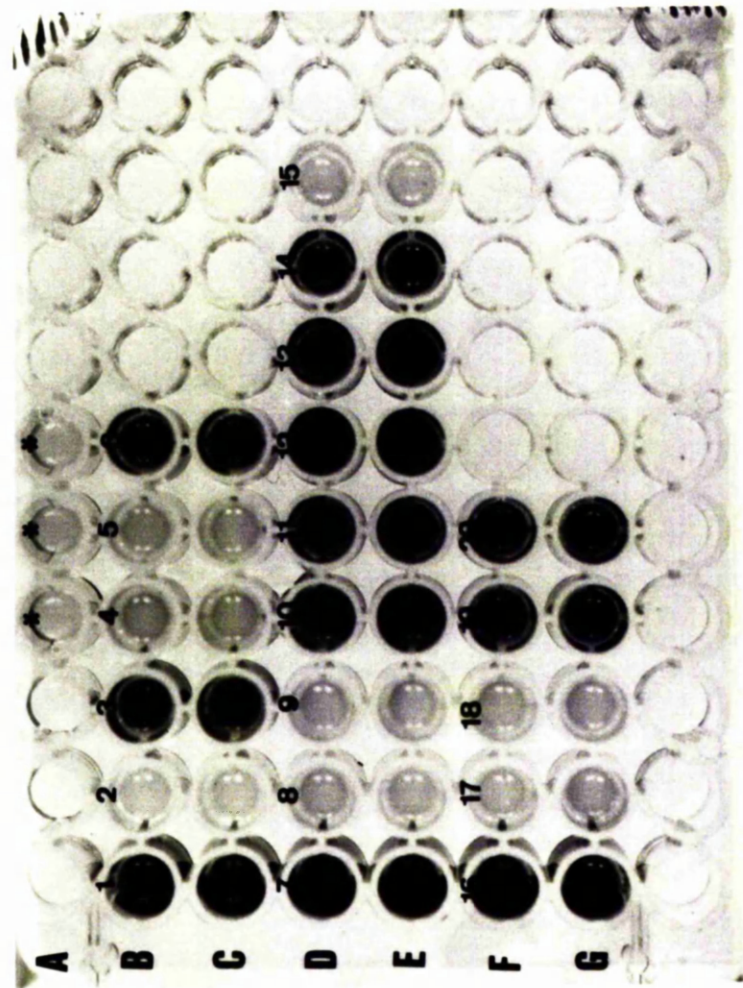
These results implied that the *B.pertussis vir* locus contained in clone pDM1 could restore expression of virulence-associated factors to avirulent phase III *B.bronchiseptica* strains.

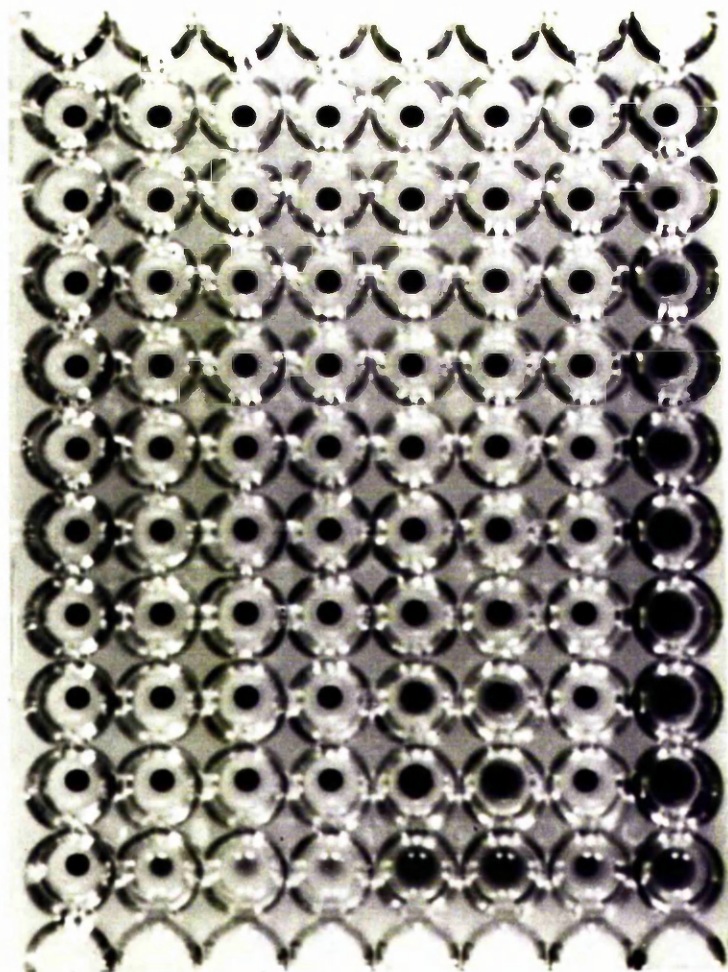
Figure 4.24.

Assay for production of AC by *B.bronchiseptica* phase III strains containing pDM1.

Production of AC was assayed by the development of a blue colour from X-gal in the wells of a microtitre tray, as described in Section 3.3.11. Samples were set up in duplicate (rows B & C, rows D & E, and rows F & G), as indicated below. The wells marked by an asterisk in row A were controls to which no *B.bronchiseptica* sample was added. For reference, the haemolytic activity of each original colony is given in brackets below.

1. *B.pertussis* L84 I [H+]
2. *B.pertussis* BP347 [H-]
3. *B.bronchiseptica* AS1 I [H+]
4. *B.bronchiseptica* AS1 III [H-]
5. *B.bronchiseptica* AS1 III (pLAFR1) [H-]
6. *B.bronchiseptica* AS1 III (pDM1) [H+]
  
7. *B.bronchiseptica* 276 I [H+]
8. *B.bronchiseptica* 276 III [H-]
9. *B.bronchiseptica* 276 III (pLAFR1) [H-]
10. *B.bronchiseptica* 276 III (pDM1) [H-]
11. *B.bronchiseptica* 276 III (pDM1) [H-]
12. *B.bronchiseptica* 276 III (pDM1) [H+]
13. *B.bronchiseptica* 276 III (pDM1) [H+]
14. *B.pertussis* L84 I [H+]
15. *B.pertussis* BP347 [H-]
  
16. *B.bronchiseptica* FW5 I [H+]
17. *B.bronchiseptica* FW5 III [H-]
18. *B.bronchiseptica* FW5 III (pLAFR1) [H-]
19. *B.bronchiseptica* FW5 III (pDM1) [H+]
20. *B.bronchiseptica* FW5 III (pDM1) [H+]





A B C D E F G H

## Section 4.5. MODULATION STUDIES.

### 4.5.1. The effect of environmental stimuli on BP347 (pRMB2) and BP347 (pDM1).

Certain chemical and physical stimuli, including MgSO<sub>4</sub>, nicotinic acid and low temperatures (25°C), result in the concomitant loss of expression of virulence determinants in *B.pertussis* in a phenomenon called antigenic modulation. Production of virulence determinants in *B.pertussis* requires the central regulatory locus, *vir*, and it was hypothesised that the effect of the modulator on expression of virulence determinants would be mediated either directly or indirectly by *vir*. The work described in this Section was performed to determine if the *vir* locus contained in clones pRMB2 and pDM1 would respond to environmental stimuli in a similar manner to a chromosomally encoded *vir* locus, and if the copy number of the clones (albeit low) would affect the response.

The effect of low temperature and nicotinic acid on expression of haemolysin was investigated. Cultures of *B.pertussis* strains BP347 (pRMB2) and BP347 (pDM1) were set up in duplicate on BG agar (+Tc). Also set up in duplicate on BG agar were cultures of *B.pertussis* phase I strains Tohama and L84 (as positive controls) and strain BP347. One set of cultures was incubated at 25°C and the other set was incubated as normal at 35°C. Cultures of all the above strains were also set up on BG agar (+/- Tc) containing 500µg/ml nicotinic acid, and incubated at 35°C. After incubation for 5 - 7 days, haemolytic activity was assessed for each culture. Results are shown in Table 4.10. Growth at low temperature resulted in loss of haemolytic activity in *B.pertussis* strains Tohama I, L84 I, BP347 (pRMB2) and BP347 (pDM1). There was no observable difference between strains with a

Table 4.10.

Influence of low temperature and nicotinic acid on haemolytic activity in BP347 (pRMB2) and BP347 (pDM1).

<i>B. pertussis</i> strain	Haemolytic activity		
	BG agar	BG agar	BG agar + nicotinic acid <sup>2</sup>
	35°C	25°C <sup>1</sup>	35°C
Tohama I	+	-	+
L84 I	+	-	-
BP347	-	-	-
BP347 (pRMB2)	+	-	+
BP347 (pDM1)	+	-	+

1. The influence of low temperature on haemolytic activity was determined by incubating cultures at 25°C.

2. The influence of nicotinic acid on haemolytic activity was determined by growing strains on BG agar containing 500 µg/ml nicotinic acid.

Tc was included in the medium for growth of strains harbouring plasmids.

chromosomally-encoded *vir* locus and strains with a plasmid encoded *vir* locus. The fact that the *vir* locus contained in clones pRMB2 and pDM1 was present in several copies had no noticeable effect on the response to the modulator. Growth in the presence of 500µg/ml nicotinic acid resulted in loss of haemolytic activity in *B.pertussis* L84 I. *B.pertussis* strains BP347 (pRMB2) and BP347 (pDM1) remained haemolytic in the presence of nicotinic acid. However *B.pertussis* Tohama I (the ancestral strain of BP347) also remained haemolytic, indicating perhaps that some *B.pertussis* strains may not modulate in the presence of nicotinic acid.

#### Section 4.6. ANALYSIS OF FHA ACTIVITY OF CLONE pRMB2.

##### 4.6.1. Analysis of *E.coli* DH1 (pRMB2) for FHA activity.

At the beginning of this project (1986), a personal communication from Dr. A. Weiss revealed that the *B.pertussis vir* and *fha* loci are located close together. Part of the initial research in this project was undertaken to determine if the *fha* locus was contained in the virulence regulatory clone pRMB2. As a preliminary investigation, a haemagglutination assay was conducted on *E.coli* DH1 (pRMB2) to determine whether pRMB2 encoded a functional FHA. *E.coli* strains DH1 and DH1 (pLAFR1) were included in the assay as negative controls. Also included were *B.pertussis* strains Tohama I, TAB I (as positive controls) and BP347 (as a negative control). Results are shown in Table 4.11. No haemagglutinating activity was detected in *E.coli* DH1 (pRMB2). The limitations of this assay were realised, and results interpreted accordingly.

Table 4.11.

Assay for production of FHA by *E. coli* DH1 (pRMB2)

Sample	Haemagglutinating activity
<i>E. coli</i> DH1 (pRMB2)	-
<i>E. coli</i> DH1	-
<i>E. coli</i> DH1 (pLAFR1)	-
<i>B. pertussis</i> Tohama I	+
<i>B. pertussis</i> TAB I	+
<i>B. pertussis</i> BP347	-

Production of FHA was assayed by haemagglutination of horse erythrocytes in the wells of a microtitre tray (as described in Section 3.3.5). Cell suspensions were serially diluted twofold from 50 µg to 0.2 µg protein per well.



#### 4.6.2. Analysis of FHA activity in *B.pertussis* strains BP353 (pRMB2) and BP353 (pDM1).

Weiss et al. (1983) reported the isolation of *B.pertussis* BP353, a Tn5 mutant deficient in FHA production. Complementation studies were performed to determine if pRMB2 (and subclone pDM1) contained all, or part of, the *fha* locus necessary to restore a functional FHA to BP353. Plasmids pRMB2, pDM1 and pLAFR1 were conjugally transferred from *E.coli* to *B.pertussis* BP353 using a tri-parental mating system. Conjugation mixes were incubated at 35°C overnight. Cells were then transferred to selective BG agar. After incubation at 35°C for 3 - 4 days, BP353 (pRMB2), BP353 (pDM1) and BP353 (pLAFR1) plates each supported growth of several hundred *B.pertussis* colonies. Donor, helper and recipient strains were shown to be unable to grow on selective BG agar, therefore all colonies obtained were presumed to have arisen as a result of plasmid acquisition.

FHA activity was assayed by haemagglutination of horse erythrocytes. The test included *B.pertussis* strains BP353 (pRMB2), BP353 (pDM1), BP353 (pLAFR1), BP353, L84 I and BP347 (the latter two strains were included as known positive and negative controls respectively). Results are shown in Figure 4.26. No haemagglutinating activity was detected in *B.pertussis* BP353, or BP353 containing the cloning vector (pLAFR1). Clone pRMB2 and subclone pDM1 did not restore any detectable FHA activity to BP353. This preliminary analysis implied that pRMB2 (and pDM1) lacked, at least, the region of the *fha* locus necessary to complement the Fha- mutation of BP353.

Figure 4.26.

Assay for production of FHA by *B.pertussis* strains BP353 (pRMB2) and BP353 (pDM1).

FHA production was assayed as described in Section 3.3.5. Cell suspensions were serially diluted 2-fold from 50µg to 0.2µg protein per well (columns 2 to 10). As a control, column 11 contains PBS + erythrocytes.

Row B: *B.pertussis* L84 I

Row C: *B.pertussis* BP353 (pRMB2)

Row D: *B.pertussis* BP353 (pDM1)

Row E: *B.pertussis* BP353 (pLAFR1)

Row F: *B.pertussis* BP353

Row G: *B.pertussis* BP347



## Section 4.7. ACTIVITY OF VIR IN E.COLI.

### 4.7.1. Transfer of pRMB2 to *E.coli* JA221 (pIL22) and analysis of AGG2 activity by slide agglutination.

Using an oligonucleotide probe complementary to the beginning of the gene encoding the serotype 2 (ST2) fimbrial subunit of *B.pertussis* Tohama I, Livey *et al.* (1987) identified a clone (pIL22) from a gene library of *B.pertussis* Wellcome 28 DNA cloned in pBR328. The aim of this work was to determine if the ST2 fimbrial subunit gene (*fim2*) was expressed in *E.coli* containing both pIL22 and the *vir* locus.

Several hundred colonies were obtained on selective agar (nutrient agar + Ap + Tc) when clone pRMB2 was conjugally transferred from *E.coli* DH1 to *E.coli* JA221 (pIL22) using a tri-parental mating system which included the use of a helper plasmid (pRK2013). Donor, helper and recipient *E.coli* strains used as controls were unable to grow on selective agar, indicating that the colonies obtained contained both pIL22 and pRMB2. Plasmid DNA was prepared from two of the *E.coli* JA221 (pIL22, pRMB2) transconjugant colonies, digested with *EcoRI*, and subjected to electrophoresis together with duplicate samples of *EcoRI*- digested pIL22 and pRMB2 DNA (Figure 4.27). Inspection of the gel confirmed that *E.coli* JA221 (pIL22, pRMB2) colonies contained both plasmids. (pIL22 does not contain any *EcoRI* restriction sites.)

In order to determine whether the plasmid encoded *vir* locus of pRMB2 regulated expression of agglutinogens, a series of slide agglutination tests using polyclonal antisera "Preston 1" and "Preston 2" was performed for *B.pertussis* strains BP347 (pRMB2), Tohama I and BP347 (see Table 4.12). *B.pertussis* Tohama I (the ancestral strain of BP347) was positive, and

Figure 4.27.

Electrophoresis of *EcoRI*-digested plasmid DNA from *E.coli* JA221 (pIL22, pRMB2).

Ethidium bromide stained gel showing *EcoRI*-digested plasmid DNA from two *E.coli* JA221 (pIL22, pRMB2) transconjugant colonies and duplicate samples of *EcoRI*-digested pRMB2 and pIL22 DNA. Numbers on the right refer to the sizes (kb) of *HindIII*-digested  $\lambda$  DNA.

Lanes 1 & 2: pRMB2

Lanes 3 & 4: pIL22

Lanes 5 & 6: plasmid DNA from *E.coli* JA221 (pIL22, pRMB2)

Lane 7: *HindIII*-digested  $\lambda$  DNA

1 2 3 4 5 6 7

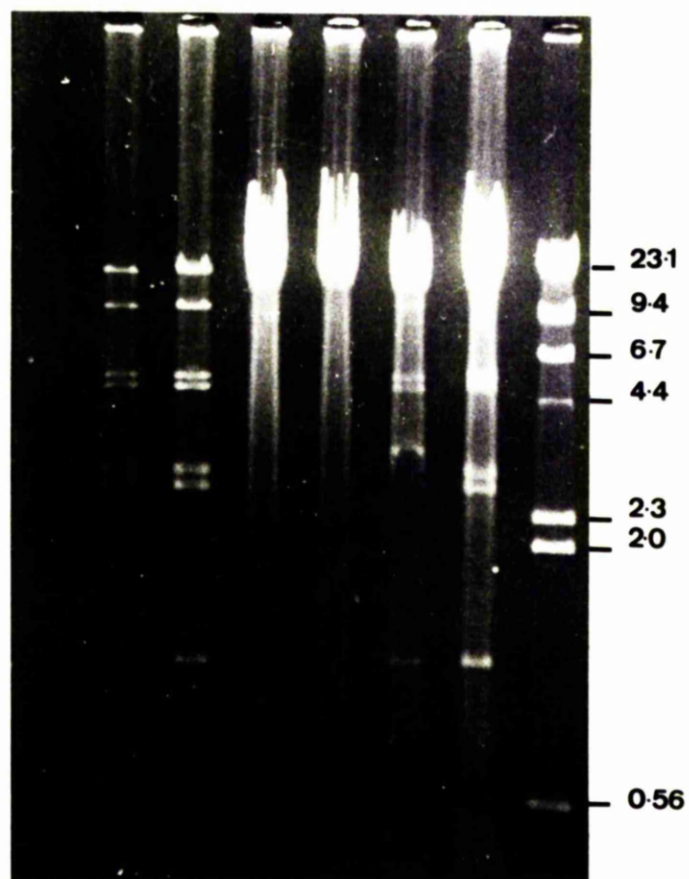


Table 4.12.

Assay for agglutigen production by slide agglutination

Sample	agglutination	
	"Preston 1"	"Preston 2"
<i>B. pertussis</i> Tohama I	+	+
<i>B. pertussis</i> BP347	-	-
<i>B. pertussis</i> BP347 (pRMB2)	+	-
<i>B. pertussis</i> BP353	+	-
<i>E. coli</i> JA221 (pIL22, pRMB2)	n.t.	-
<i>E. coli</i> JA221 (pIL22)	n.t.	-
<i>E. coli</i> DH1 (pRMB2)	n.t.	-

Slide agglutination tests were performed as described in Section 3.3.6, using polyclonal antisera "Preston 1" and "Preston 2" to detect AGG1 and AGG2 activity respectively.

n.t. = not tested

BP347 was negative for production of AGG1 and AGG2. Expression of AGG1, but not AGG2, was detected in BP347 (pRMB2). However slide agglutination analysis showed that BP353, an FHA deficient Tn5 mutant created at the same time as BP347, was also positive for AGG1 but negative for AGG2 activity (see Table 4.12). Since the *fim2* gene in pIL22 was derived from strain Wellcome 28 which expressed this AGG, it was presumed that pRMB2 would *trans*-activate the *fim2* gene encoded by pIL22.

A slide agglutination assay was performed to determine whether pRMB2 could *trans*-activate the cloned *B.pertussis fim2* gene in *E.coli*, and produce mature fimbrial proteins (see Table 4.12). No agglutination reaction with polyclonal "Preston 2" antiserum was detected in control strains JA221 (pIL22) and DH1 (pRMB2), or in the test strain JA221 (pIL22, pRMB2).

#### 4.7.2. Analysis of AGG2 expression in *E.coli* JA221 (pIL22, pRMB2) by ELISA and immunoblotting.

The ability of pRMB2 to *trans*-activate the cloned ST2 fimbrial subunit gene in *E.coli* JA221 (pIL22, pRMB2) was determined by an enzyme-linked immunosorbent assay (ELISA) using lysed whole cell samples on nitrocellulose membrane, as described in Section 3.3.4. *B.pertussis* Tohama I was included as a positive control, and *E.coli* strains JA221 (pIL22) and DH1 (pRMB2) were included as negative controls. Two primary antibodies were used for immunological detection of AGG 2: monoclonal antibody (MAb) Ag2A and polyclonal antibody "Preston 2". Results are shown in Table 4.13. Production of AGG2 was detected only in the *B.pertussis* Tohama I sample, when MAb Ag2A was used. "Preston 2" antiserum gave a strong positive reaction with the *B.pertussis* Tohama I sample, and also a positive reaction



Table 4.13.

Analysis of AGG2 expression in *E. coli* JA221 (pIL22, pRMB2)  
by enzyme-linked immunosorbent assay (ELISA).

Sample	antibody	
	Ag2A	"Preston 2"
<i>B. pertussis</i> Tohama I	+	++
<i>E. coli</i> JA221 (pIL22, pRMB2)	-	+
<i>E. coli</i> JA221 (pIL22)	-	+
<i>E. coli</i> DH1 (pRMB2)	-	+

ELISA was performed using lysed whole cell samples on nitrocellulose, as described in Section 3.3.4. Monoclonal antibody Ag2A and polyclonal "Preston 2" were used as primary antibodies and binding was detected using anti-mouse or anti-rabbit IgG horse-radish peroxidase conjugate respectively, and substrate 3-amino-9-ethylcarbazole.

The degree of colour development was scored as follows :

- negative
- + positive
- ++ strongly positive

with all three *E.coli* samples.

Expression of the *B.pertussis* *fim2* gene in *E.coli* JA221 (pIL22, pRMB2) was also assayed by immunoblotting. Figure 4.28 shows protein profiles obtained when whole cell samples of *B.pertussis* Tohama I and *E.coli* strains JA221 (pIL22, pRMB2), JA221 (pIL22) and DH1 (pRMB2) were subjected to SDS-PAGE. The protein profile of *E.coli* JA221 (pIL22, pRMB2) was examined for the presence of any additional band which may have been due to expression of the ST2 fimbrial subunit. No discernible difference was observed between the protein profiles of *E.coli* strains JA221 (pIL22, pRMB2) and JA221 (pIL22). However both these strains exhibited a prominent band not present in the *E.coli* DH1 (pRMB2) sample.

Samples were again subjected to SDS-PAGE, and the gel was immunoblotted onto nitrocellulose. The blot was incubated with "Preston 2" antiserum. Binding of antibody to the nitrocellulose was detected using anti-rabbit IgG horse-radish peroxidase conjugate and 4-chloronaphthol reagent. The resulting blot is shown in Figure 4.29. The mature AGG2 subunit is reported to have an SDS-PAGE value of 22.5kD (Irons *et al.*, 1985). The major band detected in the *B.pertussis* Tohama I sample was presumed to be the 22.5kD AGG2 subunit, however several other bands were also detected. From the deduced amino acid composition, Livey *et al.* (1987) calculated the molecular weight of the mature ST2 fimbrial subunit to be 19.2kD. There was no evidence of expression of the ST2 fimbrial subunit in the *E.coli* sample containing both pIL22 and pRMB2 clones. A band of approximately 35kD was detected in all three *E.coli* samples.

Figure 4.28.

SDS-PAGE analysis of *E.coli* JA221 (pIL22, pRMB2).

Whole-cell samples were treated and subjected to electrophoresis as described in Sections 3.3.1 and 3.3.2. Protein bands were visualised by Coomassie Blue staining. Numbers on the left refer to the sizes (kD) of molecular weight markers.

Lane 1: Molecular weight markers (SDS-6, Sigma)

Lane 2: *E.coli* JA221 (pIL22, pRMB2)

Lane 3: *E.coli* JA221 (pIL22)

Lane 4: *E.coli* DH1 (pRMB2)

Lane 5: *B.pertussis* Tohama I

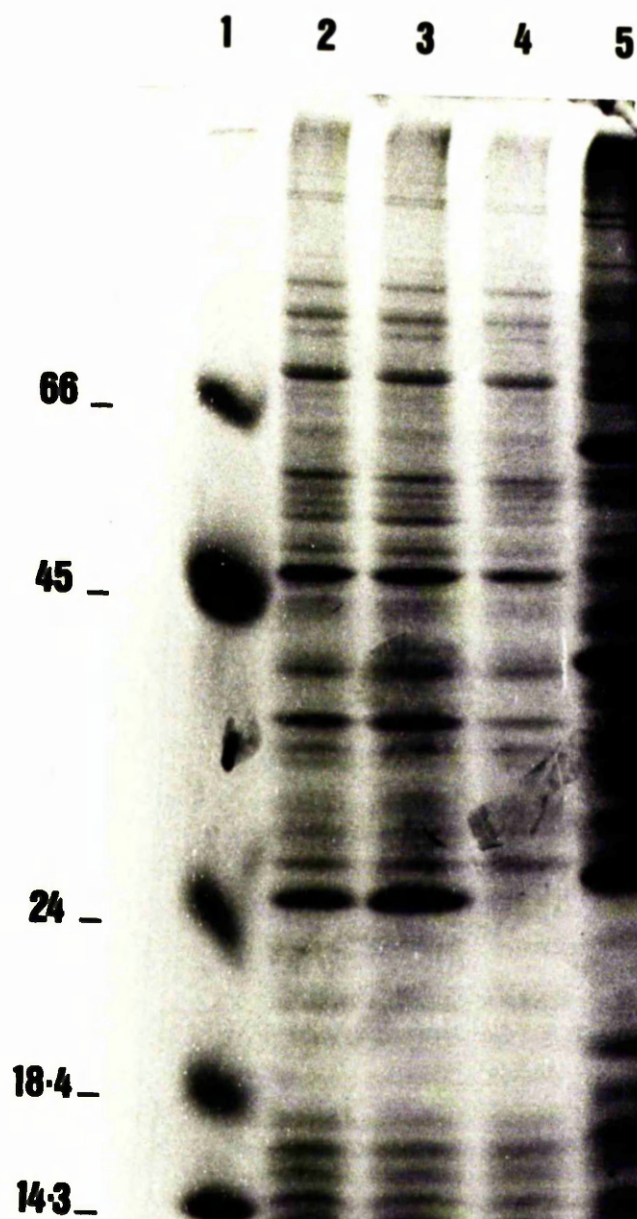


Figure 4.29.

Immunoblot analysis of *E.coli* JA221 (pIL22, pRMB2) using "Preston 2" antiserum.

After SDS-PAGE, samples were immunoblotted as described in Section 3.3.3. The filter was incubated with "Preston 2" antiserum, and antibody binding was detected using anti-rabbit IgG horse-radish peroxidase conjugate and 4-chloronaphthol reagent. The arrow marks the position of the major band (presumed to be the AGG2 subunit) observed in the *B.pertussis* Tohama I sample. Numbers on the left refer to the sizes (kD) of molecular weight markers (SDS-6H, Sigma).

Lane 1: *E.coli* JA221 (pIL22, pRMB2)

Lane 2: *E.coli* JA221 (pIL22)

Lane 3: *E.coli* DH1 (pRMB2)

Lane 4: *B.pertussis* Tohama I

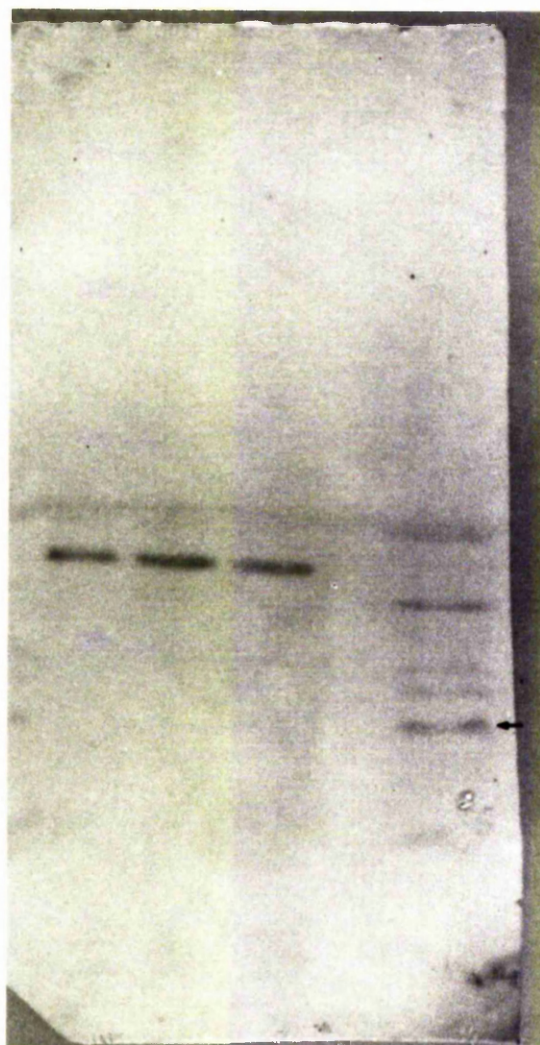
**1      2      3      4**

**116 —**  
**974 —**

**66 —**

**45 —**

**29 —**



#### 4.7.3. Construction of pDM1 subclones and their transfer to *E.coli* DH1 (p26).

A clone (p26) containing the AGG3 genetic determinant was identified from a gene library of *B.pertussis* TAB I DNA (R. Brownlie, personal communication). Clone p26 has been shown to express AGG3 in *B.pertussis*, and is dependent on a functional *vir* locus for expression (D. MacGregor, personal communication). The aim of this work was to determine if AGG3 was expressed in *E.coli* containing both p26 and the *vir* locus.

Clone p26 is a pLAFR1 derivative, and it was therefore necessary to subclone the *vir* locus into a plasmid which would make it compatible. The restriction map of pDM1 (Section 4.2.3) shows that the region essential for regulation of expression of virulence factors in *B.pertussis* is contained within an 8kb fragment. A cloning experiment was performed to obtain a subclone of pDM1 containing the 8kb *Bam*HI to *Eco*RI fragment in vector pIC20H.

Plasmid pIC20H was digested with both *Eco*RI and *Bam*HI and subjected to electrophoresis. Vector DNA was purified from the gel using the SS-BIOTRAP apparatus, and dephosphorylated. For preparation of insert DNA, pDM1 was first digested to completion with *Bam*HI, phenol extracted and ethanol precipitated. Recovered *Bam*HI-digested pDM1 DNA was then partially digested with *Eco*RI, using conditions which had previously been found to generate a significant proportion of fragments in the 8kb range. The sample was subjected to electrophoresis and, using the SS-BIOTRAP apparatus, DNA was recovered from a gel slice cut to include fragments of around 8kb. Dephosphorylated vector 10ng and insert DNA 100ng were ligated together for 2h and transformed into freshly prepared *E.coli* DH5 $\alpha$  cells. As controls, 10ng of ligated, dephosphorylated vector DNA, and samples containing 1ng

and 10ng of pIC20H plasmid DNA were also transformed. Transformants were selected on nutrient agar containing Ap + IPTG + X-gal.

The 10ng sample of pIC20H DNA resulted in semi-confluent growth of blue colonies and the 1ng sample resulted in 354 blue colonies, giving a transformation efficiency of approximately  $3.5 \times 10^5$  transformants per  $\mu\text{g}$  DNA. As expected, no colonies were obtained for dephosphorylated vector DNA. Transformation of purified pDM1 fragments ligated with vector DNA resulted in 41 white colonies, presumed to contain recombinant DNA. Plasmid DNA was prepared from 20 white colonies, linearised by *Bam*HI digestion, and analysed by electrophoresis. Eighteen samples contained a band of 8kb, one sample contained a band of 10.7kb, and the remaining sample contained a band of 6.6kb. These three clone types were designated pDM62, pDM623 and pDM14 respectively. Since pIC20H vector is 2.7kb, the sizes of the genomic inserts were calculated to be 5.3kb (pDM62), 8kb (pDM623) and 3.9kb (pDM14). Figure 4.30 shows the restriction profiles obtained when clones pDM62, pDM623 and pDM14 were digested with *Bam*HI and *Eco*RI. This enabled the region of pDM1 contained within each clone to be determined, as shown in Figure 4.31. Clone pDM623 contains the region essential for regulation of expression of virulence factors in *B.pertussis*.

To determine if pDM623 would allow expression of the AGG3 genetic determinant in *E.coli*, competent *E.coli* DH1 (p26) cells were transformed with a 10ng sample of pDM623 plasmid. Samples 10ng of plasmids pDM30 and pDM62 (both of which contain a part of the *vir* locus) were also transformed. Transformants were selected on nutrient agar containing Tc + Ap. Transformation by all three plasmids resulted in semi-confluent growth. *E.coli* DH1 (p26) was unable to grow on selective agar, therefore all colonies obtained were presumed to have arisen as a result of acquisition



Figure 4.30.

Restriction enzyme profiles of pDM1 subclones.

Ethidium bromide stained gel showing samples of clones pDM62, pDM623 and pDM14 digested with *Bam*HI and *Eco*RI. Samples of pIC20H and pDM30 digested with *Bam*HI and *Eco*RI were included for reference. Numbers on the right refer to the sizes (kb) of *Hind*III-digested  $\lambda$  DNA.

Lane 1: *Bam*HI-digested pIC20H  
Lane 2: *Bam*HI-digested pDM30  
Lane 3: *Bam*HI-digested pDM14  
Lane 4: *Bam*HI-digested pDM62  
Lane 5: *Bam*HI-digested pDM623  
Lane 6: *Eco*RI-digested pIC20H  
Lane 7: *Eco*RI-digested pDM30  
Lane 8: *Eco*RI-digested pDM14  
Lane 9: *Eco*RI-digested pDM62  
Lane 10: *Eco*RI-digested pDM623  
Lane 11: *Hind*III-digested  $\lambda$  DNA  
Lane 12: *Pst*I-digested  $\lambda$  DNA

1 2 3 4 5 6 7 8 9 10 11 12



— 23·1  
— 9·4  
— 6·7  
— 4·4

— 2·3  
— 2·0

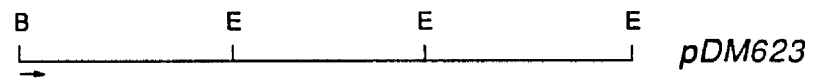
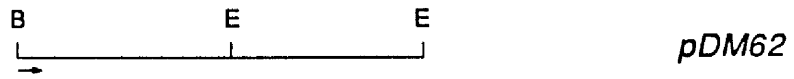
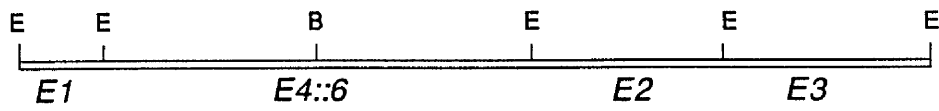
Figure 4.31.

Restriction map of various subclones of pDM1.

The thick bar represents the genomic insert of pDM1. Subclones pDM62, pDM623 and pDM14 were constructed by cloning *Bam*HI to *Eco*RI fragments of pDM1 into pIC20H. The arrows indicate the direction of transcription for the *lac* promoter of pIC20H. For reference, pDM30 (the E3 fragment cloned in pIC20H) is also shown. The direction of transcription was not determined for pDM30.

E, *Eco*RI; B, *Bam*HI.

1kb 



of the *vir* subclones. Plasmid DNA was prepared from two colonies picked from each plate, digested with *Bam*HI and subjected to electrophoresis together with *Bam*HI-digested samples of each plasmid i.e. p26, pDM30, pDM62 and pDM623. The ethidium bromide stained gel is shown in Figure 4.32. This confirmed that *E.coli* DH1 (p26) transformant colonies contained the appropriate *vir* subclone.

#### 4.7.4. Analysis of expression of the AGG3 genetic determinant in *E.coli* by immunoblotting.

Figure 4.33 shows the protein profiles obtained when whole cell samples of *B.pertussis* Wellcome 28 (serotype 1,2,3), *E.coli* DH1 (p26), *E.coli* DH1 (p26, pDM30), *E.coli* DH1 (p26, pDM62) and *E.coli* DH1 (p26, pDM623) were subjected to SDS-PAGE. No additional band which may have been due to expression of the AGG3 genetic determinant was detected when the protein profile of *E.coli* DH1 (p26, pDM623) was compared with the negative control, *E.coli* DH1 (p26).

Samples were again subjected to SDS-PAGE, and the gel was immunoblotted onto nitrocellulose. The filter was incubated with polyclonal "Preston 3" antiserum. Antibody binding was detected using anti-rabbit IgG horse-radish peroxidase conjugate and 4-chloronaphthol reagent. Inspection of the resulting blot (not presented) showed that two bands were present in the 20- 25kb region in the positive control sample, *B.pertussis* Wellcome 28, which produces AGG3. No binding of antibody was detected in any of the *E.coli* samples.

Figure 4.32.

Electrophoresis of *Bam*HI-digested plasmid DNA from *E.coli* DH1 (p26) containing various *vir* subclones.

Ethidium bromide stained gel showing *Bam*HI-digested plasmid DNA from two *E.coli* DH1 (p26, pDM30), two *E.coli* DH1 (p26, pDM62) and two *E.coli* DH1 (p26, pDM623) transformant colonies together with *Bam*HI-digested DNA from p26, pDM30, pDM62 and pDM623 plasmids. Numbers on the left refer to the sizes (kb) of *Hind*III-digested  $\lambda$  DNA fragments.

Lane 1: *Hind*III-digested  $\lambda$  DNA

Lane 2: *Pst*I-digested  $\lambda$  DNA

Lanes 3 & 4: *Bam*HI-digested plasmid DNA from *E.coli* DH1 (p26, pDM30)

Lanes 5 & 6: *Bam*HI-digested plasmid DNA from *E.coli* DH1 (p26, pDM62)

Lanes 7 & 8: *Bam*HI-digested plasmid DNA from *E.coli* DH1 (p26, pDM623)

Lane 9: *Bam*HI-digested p26

Lanes 10 & 11: *Bam*HI-digested pDM30

Lanes 12 & 13: *Bam*HI-digested pDM62

Lane 14: *Bam*HI-digested pDM623 (a very faint band of 10.7kb)



Figure 4.33.

SDS-PAGE analysis of *E.coli* DH1 (p26) containing various *vir* subclones.

Whole-cell samples were treated and subjected to electrophoresis as described in Sections 3.3.1 and 3.3.2. Protein bands were visualised by Coomassie Blue staining. Numbers on the right refer to the sizes (kD) of molecular weight markers.

Lane 1: *B.pertussis* Wellcome 28

Lane 2: *E.coli* DH1 (p26)

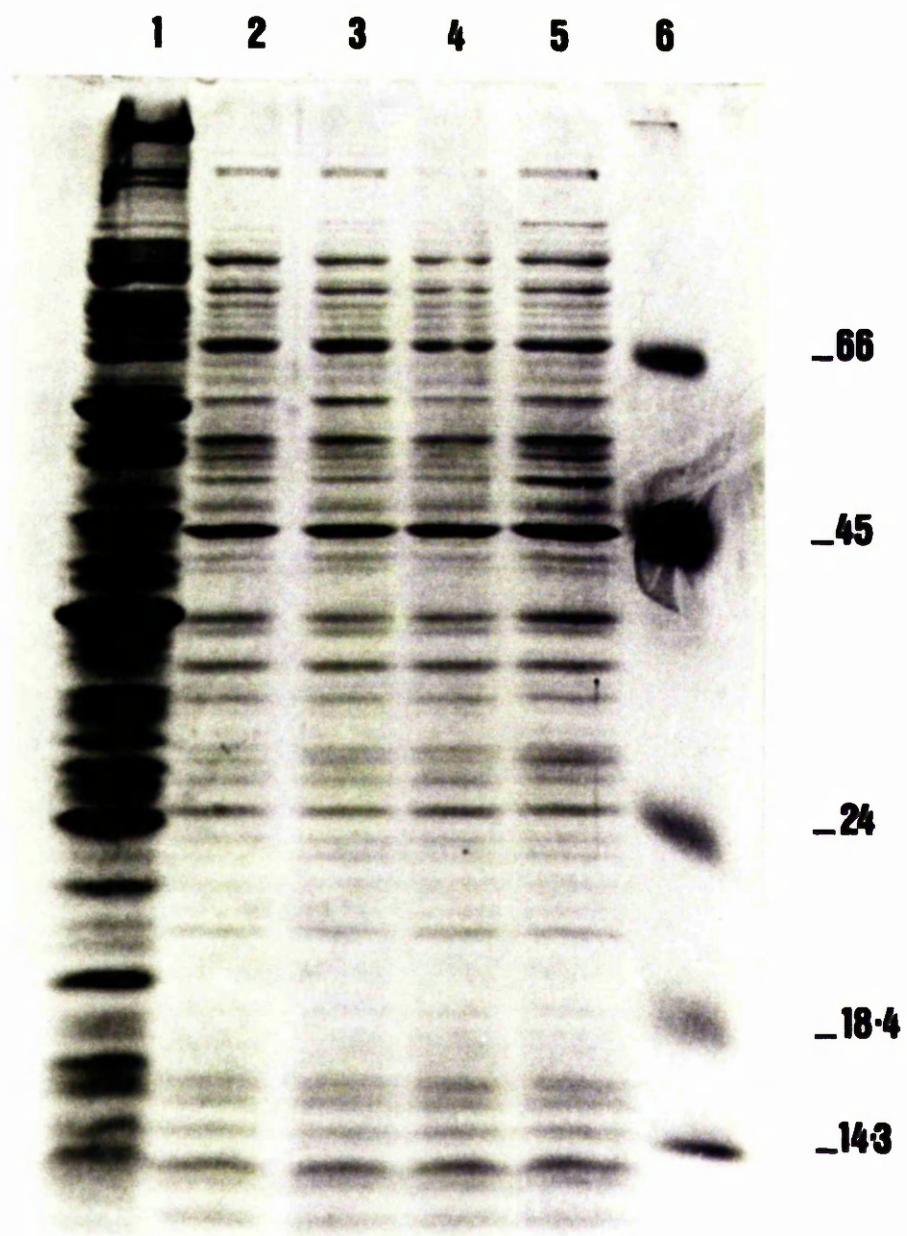
Lane 3: *E.coli* DH1 (p26, pDM30)

Lane 4: *E.coli* DH1 (p26, pDM62)

Lane 5: *E.coli* DH1 (p26, pDM623)

Lane 6: Molecular weight markers (SDS-6, Sigma)





#### 4.7.5. Analysis of expression of the AC genetic determinant in *E.coli*.

Brownlie *et al.* (1986) isolated a clone (pRMB1) containing the AC and HLY genetic determinants, from a gene library of *B.pertussis* DNA cloned in pLAFR1. However, neither AC or HLY activities were expressed in *E.coli* harbouring pRMB1 (Brownlie *et al.*, 1988). As this may have been due to the requirement for *vir*, the aim of this work was to analyse AC activity in a  $\Delta$ *cya* strain of *E.coli*, CAA8306, containing compatible plasmid clones of the AC genetic determinant and the *vir* locus.

Figure 4.34 shows a restriction map of pRMB1 and its various subclones. Expression of the *cya* gene from the *lac* promoter contained in pIC20H was detected from clones pRMB3 and pRMB9 contained in *E.coli* CAA8306 (Brownlie *et al.*, 1988). Clones pRMB11 and pRMB12 contain a 6kb *Cla*I fragment from pRMB1 cloned in opposite orientations in vector pIC20H. AC activity was detected in *E.coli* CAA8306 harbouring pRMB12, but not pRMB11, suggesting that AC activity was only detected when the *cya* gene was expressed from the *lac* promoter of pIC20H (R. Brownlie, personal communication). However, preliminary data suggested that AC activity (which was calmodulin responsive) could be detected in *E.coli* CAA8306 harbouring pRMB11 when the *vir* locus was provided in-trans (R. Brownlie, personal communication). To follow up and extend this line of investigation, *E.coli* CAA8306 was transformed with various combinations of plasmids containing the *cya* gene and *vir*.


Competent *E.coli* CAA8306 cells were first transformed with 10ng samples of the broad host-range plasmids i.e. pRMB2, pDM1 and, as a negative control, pLAFR1. Transformants were selected on nutrient agar containing Tc. Less than 10 colonies were obtained from each transformation. Since *E.coli* CAA8306 was <sup>S</sup>Tc<sup>r</sup>, all colonies were presumed to contain the

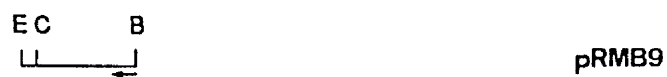
Figure 4.34.

**Restriction map of pRMB1 and its various subclones.**

The thick bar represents the genomic insert of pRMB1, which contains the *B.pertussis* AC and HLY genetic determinants cloned in pLAFR1. The direction of transcription of the AC/HLY operon, as shown here, is from right to left. The hatched area represents the length of the protein coding region. Subclones were constructed in vector pIC20H, and the arrows indicate the direction of transcription from the *lac* promoter of pIC20H. Data shown here was obtained from R. Brownlie (personal communication) and Rogel *et al.* (1989).

E, *EcoRI*; B, *BamHI*; C, *ClaI*.

1kb 



appropriate plasmid.

Competent *E.coli* CAA8306 (pRMB2), *E.coli* CAA8306 (pDM1) and *E.coli* CAA8306 (pLAFR1) cells were then separately transformed with 10ng samples of plasmids pRMB11 and pRMB12. Several hundred transformants, selected on nutrient agar containing Tc plus Ap, were obtained in each case.

Competent cells of *E.coli* CAA8306 (pRMB1) were prepared. These cells were transformed with 10ng samples of pDM623 (which contains the *vir* locus), pDM62 and pDM30 (which each contain a part of the *vir* locus) and pDM14 (as a negative control). Several hundred transformant colonies, selected on nutrient agar containing Tc plus Ap, were obtained in each instance.

To create the appropriate control strains, competent *E.coli* CAA8306 cells were transformed with 10ng samples of pDM623, pDM62, pDM30, pDM14, pRMB11 and pRMB12. Several hundred colonies, selected on nutrient agar containing Ap, were obtained from transformation with all plasmids except pDM14 (different DNA preparations of pDM14 were used to transform *E.coli* CAA8306 (pRMB1) and *E.coli* CAA8306).

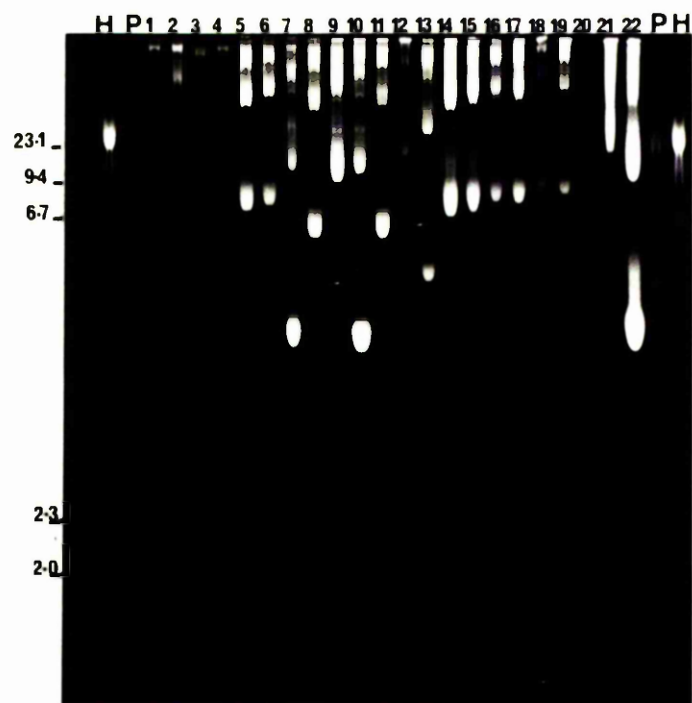
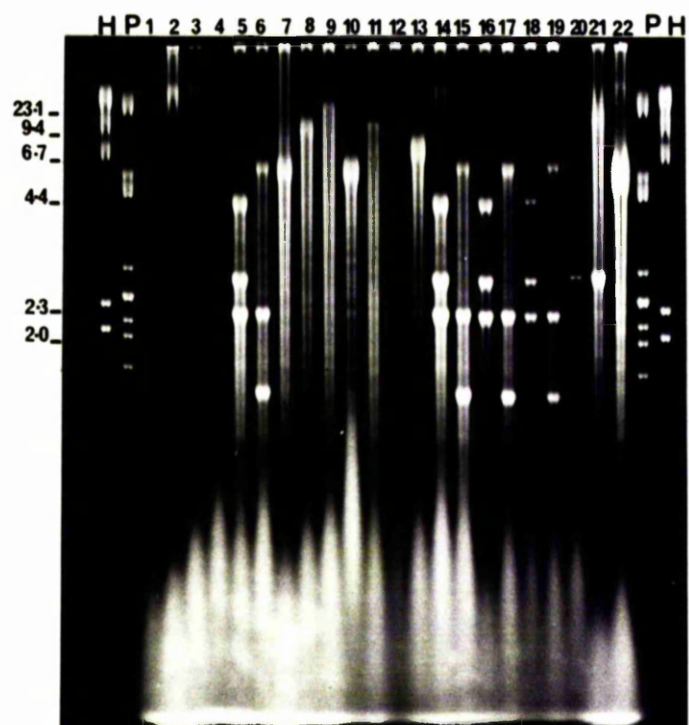
Although antibiotic resistance of *E.coli* CAA8306 transformant colonies suggested that they contained the appropriate plasmid(s), plasmid DNA was prepared from several colonies selected from each transformant plate, and analysed. Figure 4.35 shows representative samples of undigested and *Bam*HI-digested plasmid DNA after electrophoresis. The yield of DNA for the low copy-number plasmids (pRMB1, pRMB2, pDM1 and pLAFR1) was extremely low, and was best visualised in undigested samples from *E.coli* CAA8306 harbouring only these plasmids (i.e. the strains which were used for subsequent transformation by the pIC20H clones). The yield of DNA for the high copy-number pIC20H clones was high, and the presence of the correct pIC20H clone

Figure 4.35.

Electrophoresis of undigested and *Bam*HI-digested plasmid DNA from *E.coli* CAA8306 containing various *cya* and *vir* clones.

Ethidium bromide stained gels showing undigested and *Bam*HI-digested plasmid DNA from a variety of *E.coli* CAA8306 transformant colonies and from *E.coli* CAA8306 (pRMB1), *E.coli* CAA8306 (pIC20H), *E.coli* CAA8306 (pRMB3) and *E.coli* CAA8306 (pRMB9). (A) contains undigested and (B) contains *Bam*HI-digested samples. (Plasmid preparations shown here were not RNase-treated.) Numbers on the left refer to the sizes (kb) of *Hind*III-digested  $\lambda$  DNA.

H: <i>Hind</i> III-digested $\lambda$ DNA	11: pRMB1, pDM62
P: <i>Pst</i> I-digested $\lambda$ DNA	12: pRMB1, pDM623
1: pRMB1	13: pRMB1, pDM14
2: pLAFR1	14: pLAFR1, pRMB11
3: pDM1	15: pLAFR1, pRMB12
4: pRMB2	16: pDM1, pRMB11
5: pRMB11	17: pDM1, pRMB12
6: pRMB12	18: pRMB2, pRMB11
7: pDM30	19: pRMB2, pRMB12
8: pDM62	20: pIC20H
9: pDM623	21: pRMB3
10: pRMB1, pDM30	22: pRMB9

**A****B**

was confirmed in each instance.

In a preliminary analysis, AC activity in *E.coli* CAA8306 harbouring the *B.pertussis cya* gene and *vir* locus cloned in compatible plasmids (pRMB1 and pDM623 respectively) was determined. Samples of *E.coli* CAA8306 harbouring both pRMB1 and pDM62, pDM30 or pDM14 were included in the assay, together with *E.coli* CAA8306 harbouring only pDM623, pDM62 or pDM30 as negative controls. To minimize the loss of cultures during transportation, two cultures of each strain were sent to Dr. E. Hanski at The Weizmann Institute of Science, Israel, for analysis of AC activity (Table 4.14). Results were somewhat surprising, as the level of AC activity for the two cultures widely differed in each case e.g. the values obtained for *E.coli* CAA8306 (pDM30) were 29.03 and 0.54 nmol cAMP per min per mg protein. The cultures used for the assay were grown from two separate colonies selected from each transformation plate, and one would not have expected to find a difference between the individual colonies. (Plasmid analysis confirmed that cultures contained the appropriate plasmid(s).) The two cultures of *E.coli* CAA8306 harbouring both pRMB1 and pDM623 (the latter contains the whole *vir* locus) produced values of 12.76 and 56.87 nmol cAMP per min per mg protein, which appeared to be slightly higher than values obtained for the other strains. However, as this assay did not contain any known positive and negative controls, the enzymic activity of *B.pertussis* AC in *E.coli* was measured for a more extensive range of samples.

The ability of various *vir* clones to *trans*-activate the AC genetic determinant of clones pRMB1, pRMB11 and pRMB12 in *E.coli* CAA8306 was investigated. The assay involved two steps: incubation of the sample with ATP, the substrate from which the adenylate cyclase generates cAMP, and then measurement of the cAMP generated with a cAMP assay kit (Amersham, see



Table 4.14.

Adenylate cyclase enzymic activity in *E. coli* CAA8306  
harbouring pRMB1 plus various *vir* subclones

plasmid(s)	Enzymic activity	
	nmol cAMP per min per mg protein culture a	per mg protein culture b
pDM30	29.03	0.54
pDM62	0.67	0.54
pDM623	1.68	0.16
pRMB1, pDM14	9.05	1.28
pRMB1, pDM30	22.92	0.65
pRMB1, pDM62	2.94	0.05
pRMB1, pDM623	12.76	56.87

Data was obtained from Dr. E. Hanski. The assay was performed in the presence of calmodulin, as described by Brownlie *et al.* (1988).

The two cultures (a and b) used for each strain were grown from separate transformant colonies.

Section 3.1.11). *E.coli* CAA8306 (pRMB3) and *E.coli* CAA8306 (pRMB9) were included in the assay as known positive controls. Samples of *E.coli* CAA8306 harbouring single plasmids i.e. vectors, *vir* clones or *cya* clones were also included in the assay. Each sample was assayed in duplicate, and the two values obtained in each case were very similar. Results are shown in Table 4.15.

Positive control samples, *E.coli* CAA8306 (pRMB3) and *E.coli* CAA8306 (pRMB9) had an enzymic activity higher than the uppermost limit of cAMP per assay tube detectable by the cAMP assay kit i.e. greater than 16 picomoles cAMP per incubation tube. AC enzymic activity was detected in samples harbouring pRMB11 and pRMB12, however the level of activity was considerably lower than for the positive control samples. The presence of the *vir* locus *in-trans* had no marked effect on AC activity in *E.coli*.

Table 4.15

Adenylate cyclase enzymic activity in *E. coli* CAA8306 containing various AC and *vir* clones

Plasmid(s)	Characteristics	Enzymic activity	
		pmol cAMP per min per incubation tube	nmol cAMP per min per mg protein
pLAFR1	Broad-host-range cloning vector compatible with pIC20H.	0.1	5.7
pRMB1	A pLAFR1 derivative containing the <i>B. pertussis</i> AC genetic determinant.	0.3	9.5
pRMB2	A pLAFR1 derivative containing the <i>B. pertussis</i> <i>vir</i> locus.	0.1	4.4
pDM1	A subclone of pRMB2 containing the <i>B. pertussis</i> <i>vir</i> locus.	0.0	0.0
pIC20H	A high copy-number cloning vector compatible with pLAFR1.	0.2	8.1
pRMB11	A pIC20H derivative containing the 6kb <i>ClaI</i> fragment of pRMB1 cloned in the opposite orientation to the <i>lac</i> promoter.	0.3	10.6
pRMB12	A pIC20H derivative containing the 6kb <i>ClaI</i> fragment of pRMB1 cloned in the same orientation as the <i>lac</i> promoter.	1.9	62.2
pDM30	A pIC20H derivative containing the 2.7kb <i>EcoRI</i> fragment (E3) of pDM1 (contains part of the <i>vir</i> locus).	0.0	0.0
pDM62	A pIC20H derivative containing the 5.3kb <i>BamHI</i> to <i>EcoRI</i> fragment of pDM1 (contains part of the <i>vir</i> locus).	0.0	0.0
pDM623	A pIC20H derivative containing the 8kb <i>BamHI</i> to <i>EcoRI</i> fragment of pDM1 (contains the <i>vir</i> locus).	0.0	0.0

contd.

Table 4.15. (contd.)

Plasmid(s)	Characteristics	Enzymic activity	
		pmol cAMP per min per incubation tube	nmol cAMP per min per mg protein
pDM14	A pIC20H derivative containing the 3.9kb <i>Bam</i> HI to <i>Eco</i> RI fragment of pDM1 (negative control).	n.a.	n.a.
pRMB3	A pIC20H derivative containing the 10kb <i>Bam</i> HI fragment of pRMB1 cloned in the same orientation as the <i>lac</i> promoter.	>16.0	>290.9
pRMB9	A pIC20H derivative containing the 3kb <i>Bam</i> HI to <i>Eco</i> RI fragment of pRMB1 cloned in the same orientation as the <i>lac</i> promoter.	>16.0	>290.9
pRMB1, pDM30		0.3	8.9
pRMB1, pDM62		0.1	3.2
pRMB1, pDM623		0.2	4.5
pRMB1, pDM14		0.1	3.3
pLAFR1, pRMB11		1.7	38.0
pLAFR1, pRMB12		1.4	55.9
pDM1, pRMB11		1.1	48.5
pDM1, pRMB12		1.3	57.3
pRMB2, pRMB11		0.8	75.6
pRMB2, pRMB12		0.5	16.2

The assay was performed as described in Section 3.1.11. Samples were incubated with ATP and, using a cAMP assay kit (Amersham), the cAMP generated was determined from a calibration curve for the assay of cAMP in the range 0.2 to 16 pmoles per incubation tube. Each sample was assayed in duplicate, and the average is shown. Protein concentration was determined by the method of Bradford (1976).

n.a. = not available.

Section 5: DISCUSSION.

## Section 5.1. MOLECULAR ANALYSIS OF VIR.

### 5.1.1. Hybridisation analysis of BP347 DNA.

Since pRMB2 was able to restore a Vir+ phenotype to *B.pertussis* BP347, it was presumed that pRMB2 contained a region of DNA homologous to that which was inactivated by the Tn5 insertion in BP347 (Brownlie *et al.*, 1988). Restriction analysis of pRMB2 with *EcoRI* revealed that the genomic insert was 21.6kb, and consisted of six fragments, designated E1 (1.1kb), E2 (2.5kb), E3 (2.7kb), E4 (4.7kb), E5 (5.1kb) and E6 (10.0kb).

Genomic DNA from *B.pertussis* strains L84 I, Tohama I and TAB I hybridised to fragments E1 to E6 of pRMB2 (Section 4.1.1). There was no detectable difference in the *vir* region of these three phase I strains. Hybridisation analysis of *EcoRI*-digested BP347 DNA using pRMB2 as a probe showed that the 2.7kb fragment was missing, and exhibited instead was a fragment of 8.4kb, which was presumed to contain a copy of Tn5 (5.7kb). A high molecular weight fragment in the BP347 sample also hybridised to the pRMB2 probe. This band was of lower intensity than the other bands detected in the sample, which implied that it had arisen as a result of the probe hybridising weakly to a region elsewhere in the *B.pertussis* chromosome. The excess of DNA in the BP347 sample probably accounted for its detection in only this sample. When the E3 fragment of pRMB2 was used as a probe (Section 4.1.4), it hybridised to the 8.4kb fragment in the sample of *EcoRI*-digested BP347 DNA, confirming that the Tn5 insertion in BP347 was located in a 2.7kb *EcoRI* fragment. The inactivation of this region by Tn5 implied that it was essential to the *vir* locus.

The site of Tn5 insertion in BP347 was also determined with respect to *BamHI* fragments (Section 4.1.8). *BamHI*-digestion of pRMB2 produced six

fragments: B1 (1.4kb), B2 (2.4kb), B3 (2.7kb), B4 (2.9kb), B5 (4.8kb) and B6 (a 33.5kb fragment consisting of pLAFR1 vector and the end portions of the genomic insert of pRMB2).

*B.pertussis* strains L84 I, Tohama I and TAB I showed identical patterns of hybridisation: pRMB2 hybridised to genomic *Bam*HI fragments corresponding to B1 to B5, and also to fragments of approximately 12kb and 14kb. The latter two bands represented *Bam*HI fragments of genomic DNA to which the *Bam*HI to *Eco*RI terminal portions of the genomic insert of pRMB2 hybridised. These bands were both less intense than expected, which suggested that the high molecular weight DNA had not been transferred efficiently during the Southern blotting procedure. A band of 4.2kb was also present in all samples. The intensity of this band suggested that it was due to hybridisation of pRMB2 to a homologous region elsewhere in the chromosome, substantiating earlier findings. In BP347 DNA, the 14kb *Bam*HI fragment was missing. Since Tn5 contains a *Bam*HI site, its insertion should produce two new *Bam*HI fragments. Only one band (of around 9kb) was detected, indicating that the two fragments produced were of equal size.

Hybridisation studies using the E3 fragment as a probe confirmed that the Tn5 insertion in BP347 was located in a 14kb *Bam*HI fragment. Hybridisation data implied that the E3 fragment was located at, or near, one of the ends of the genomic insert of pRMB2. The subcloning of *vir* from pRMB2 is discussed below, however an alternative strategy would have been to isolate fragments of around 14kb from *Bam*HI-digested *B.pertussis* DNA, and clone them in a broad host-range vector. Clones could then have been transferred to Vir- BP347 for complementation analysis. The insertion of Tn5 into a 2.7kb *Eco*RI fragment and a 14kb *Bam*HI fragment in strain BP347 was later verified by comparison with a restriction map of the *vir* region

published by Stibitz *et al.* (1988a).

#### 5.1.2. Subcloning and complementation analysis of E3.

The E3 fragment of pRMB2 was subcloned in the broad host-range vector pLAFR1, and also in the high copy-number vector pIC20H. In each instance, ligation reactions contained a 10-fold molar excess of insert DNA, to favour intermolecular ligation at the expense of intramolecular ligation. To minimize self-ligation of *EcoRI*-digested vector DNA, the 5' phosphate groups were removed by treatment with CIP. Comparison of ligated, non-CIP treated vector DNA with ligated, dephosphorylated vector DNA showed that the yield of transformation for the latter was reduced by 97.3% for pLAFR1, and 98.1% for pIC20H, indicating that the CIP treatment had been highly effective in both cases. *EcoRI*-digestion of pIC20H, followed by religation, decreased the yield of transformation to 25%, compared to supercoiled pIC20H DNA. This value was lower than the expected value of >40%, based on data from Boehringer Mannheim (the manufacturers of T4 DNA ligase) for pBR322. Although antibiotic resistance of transformant colonies implied that they contained plasmid DNA, restriction analysis was used to confirm the presence of the correct recombinant clone in each case. Clones derived from pLAFR1 and pIC20H were designated pDM3 and pDM30 respectively.

Since insertion of Tn5 into the 2.7kb *EcoRI* fragment of BP347 resulted in loss of expression of virulence factors, clone pDM3 was transferred by conjugation from *E.coli* to BP347, to determine whether the E3 fragment would contain sufficient sequence to restore a Vir<sup>+</sup> phenotype to the strain. Virulence-associated factors lost by insertional inactivation of *vir* in BP347 included HLY. Therefore visualisation of haemolytic activity of transconjugant colonies on BG agar provided a convenient means of



determining whether the virulent phenotype had been restored. Results from a preliminary experiment, in which conjugation mixes were incubated overnight before selection, showed that pDM3 conferred an Hly<sup>+</sup> phenotype to only 1% of BP347 (pDM3) colonies. In a follow up experiment, in which conjugation mixes were incubated for only 8h before selection, all transconjugant colonies containing pDM3 were Hly<sup>-</sup>. BP347 (pDM3) H<sup>+</sup> strains had also regained other properties associated with virulence, such as AC, FHA and the ability to uptake CR. In contrast, BP347 (pDM3) H<sup>-</sup> strains were negative for these properties. It was concluded that the 2.7kb *EcoRI* fragment of pDM3 did not contain all of the essential *vir* region necessary for expression of virulence-associated factors in *B.pertussis*.

The fact that only a small percentage of BP347 (pDM3) H<sup>+</sup> transconjugants was obtained, suggested that they had arisen not as a result of pDM3 acting *in-trans*, but as a result of recombination in the *vir* region. The likelihood of homologous recombination occurring may have been increased by the longer incubation period of the conjugation mixes before selection. When a single BP347 (pDM3) H<sup>+</sup> colony was subcultured in the absence of Tc selection, the colonies produced were Tc<sup>s</sup> (pLAFR1 confers Tc<sup>r</sup>) and Km<sup>s</sup> (Tn5 confers Km<sup>r</sup>), but retained a Vir<sup>+</sup> phenotype. This confirmed that homologous recombination had occurred in this strain such that the copy of Tn5 was then contained within plasmid pDM3, and a fully functional *vir* locus was restored to BP347 with an attendant gain of expression of virulence-associated factors. In contrast, subculture of BP347 (pDM3) H<sup>-</sup> in the absence of Tc selection produced colonies which had lost plasmid (Tc<sup>s</sup>) but retained Tn5 (Km<sup>r</sup>). This indicated that no homologous recombination had occurred, and confirmed that pDM3 acting *in-trans* was unable to restore a Vir<sup>+</sup> phenotype to BP347.

### 5.1.3. Subcloning and complementation analysis of randomly-generated fragments of pRMB2.

The 2.7kb *EcoRI* fragment did not contain the entire *vir* sequence, therefore larger fragments of approximately 6kb, which would hopefully encompass the *vir* locus, were selected for subcloning. The restriction enzyme *Sau3A*, which has a 4bp recognition site, was used to partially digest pRMB2 DNA to generate a series of random fragments.

No recombinant clones were detected when size-selected *Sau3A* fragments were cloned in the *BamHI* site of the broad host-range vector pRK291. The lack of recombinant clones could have been due to any one (or combination) of the multitude of factors governing the processes of ligation and transformation.

The fact that transformation frequencies with larger plasmids are dramatically reduced (Hanahan, 1983) may have been a contributory factor.

Factors affecting the *in vitro* ligation of vector and donor DNAs to form chimeric DNA molecules include: the molar ratio of vector to insert DNA, the concentration of DNA in the reaction mixture and the concentration of DNA ends in the reaction mixture (see Maniatis *et al.*, 1982). Transformation of *E.coli* by plasmid DNA involves three stages: binding of DNA to the outside of the cell, transport of DNA across the cell envelope and establishment of the transforming DNA as a replicon itself, or by recombination with a resident replicon. A number of parameters influence the transformation process, such as competence of cells and concentration of DNA (reviewed by Saunders and Saunders, 1988).

The randomly-generated *Sau3A* fragments of pRMB2 were subcloned into broad host-range vector pRK310. A number of ligation reactions, which contained various ratios of vector to insert DNA, were set up to determine empirically the optimal conditions for formation of hybrid DNA molecules. Transformation by all ligation mixtures yielded recombinant colonies, easily discernible as white, on medium containing IPTG and X-gal. The ligation mixture containing 100ng of vector DNA and 50ng of insert DNA produced the highest number of recombinant molecules, and was therefore presumed to represent the optimal conditions out of those tested. Transformation efficiency for supercoiled DNA was considerably reduced (perhaps by as much as 1000-fold) for pRK310 compared with pIC20H, confirming the inverse relationship between plasmid size and transformation efficiency.

None of the 48 recombinant clones tested was able to restore an Hly+ phenotype to Vir- BP347, which implied that none of the *Sau3A* fragments cloned contained sufficient sequence to encode an active *vir* locus. Six recombinant plasmids were digested with both *EcoRI* and *HindIII* and analysed by gel electrophoresis. No fragment of 2.7kb was detected, indicating that none of the recombinant clones examined contained an insert which encompassed the 2.7kb E3 fragment which is essential to *vir*. Analysis of the purified size-selected *Sau3A* fragments used for ligation, showed that they ranged in size from 5kb to 9kb. However, the average insert size of recombinant clones was only 5.4kb, suggesting that there had been bias towards the formation of transformants containing smaller recombinant plasmids.

#### 5.1.4. Construction and complementation analysis of pDM1.

As a more direct approach, hybridisation analysis was used to identify putative *vir*-containing fragments of pRMB2. At this stage in the study, the location of the E3 fragment within the 26.1kb insert of pRMB2 was unknown, therefore a series of restriction enzyme digests of pRMB2 was hybridised with E3 in order to identify fragments which would encompass the essential E3 region, and would be large enough to potentially encode an active *vir* locus.

*Bam*HI, *Bgl*III and *Sac*I digests each contained a large fragment, which consisted of pLAFR1 and the terminal portions of the genomic insert of pRMB2, which hybridised to E3. This was consistent with hybridisation data which had indicated that E3 was located in one of the end regions of the genomic insert of pRMB2. The identification of these three large fragments was fortunate in that isolation and ligation of the fragments would result in pLAFR1 clones which could be readily transferred to BP347 for complementation analysis. The *Bam*HI fragment (designated B6) was chosen because on visual examination of an ethidium bromide stained gel it appeared larger than the *Bgl*III and *Sac*I fragments, and was therefore more likely to contain an active *vir* locus.

The B6 fragment, which consisted of pLAFR1 vector plus *Bam*HI to *Eco*RI end portions of the pRMB2 genomic insert, was ligated to give a clone designated pDM1. When transferred by conjugation from *E.coli* to BP347, pDM1 restored expression of virulence-associated determinants such as HLY, AC, FHA, PT, HLT and X-OMPs. This demonstrated that pDM1 encoded the *vir* locus. When single colonies of BP347 (pRMB2) and BP347 (pDM1) were subcultured in the absence of Tc selection, data obtained indicated that loss of plasmid was accompanied by loss of the virulent phenotype, confirming that the *vir*

locus encoded by these clones was acting *in-trans* to restore expression of virulence-associated genes to BP347.

Transposon Tn5 was used to generate a series of Vir- mutants in *B.pertussis* Wellcome 28 (M. Ward, personal communication). The *vir* region of nine of these mutants was analysed: only two contained a Tn5 insertion (in the 2.5kb *EcoRI* fragment), two contained a rearrangement (either inversion or deletion) and five showed no obvious change and may have represented avirulent phase variant strains which had arisen as a result of a spontaneous frame-shift mutation in *vir*, similar to that described by Stibitz *et al.* (1989). Clone pDM1 was able to restore a Vir+ phenotype to all nine Vir- strains (M. Ward, personal communication). These data substantiated the fact that pDM1 encoded the essential virulence region. The fact that the *vir* locus of pDM1 was derived from strain TAB I, and its product was able to confer a virulent phenotype to Vir- mutants derived from strains Tohama I and Wellcome 28, implied that these phase I strains contained a similar *vir* locus.

#### 5.1.5. Restriction enzyme mapping of the *vir* region.

Digestion of pDM1 with *EcoRI* produced four genomic fragments: E1, E2, E3 and an hybrid fragment formed by ligation of two *BamHI* to *EcoRI* fragments. Hybridisation of *EcoRI*-digested pRMB2 DNA with a pDM1 probe showed that the non-contiguous *EcoRI* fragment of pDM1 was derived from the E4 and E6 fragments of pRMB2. This fragment was therefore designated E4::6.

Restriction maps were compiled for clones pDM1 and pRMB2, for the enzymes *EcoRI*, *BamHI* and *BglIII*. Clone pDM1 contained two non-contiguous genomic DNA fragments of 3.9kb and 8.0kb. The fact that the latter fragment contained E3, indicated that a functional *vir* locus was encoded within this

8.0kb region.

For comparison of a restriction map of pRMB2 with a published map of a clone (pUW21-26) containing the *B.pertussis vir* and *fha* loci (Stibitz *et al.*, 1988a), see Figure 5.1. The two *EcoRI* fragments, E2 and E3, contained within the *vir* locus of pRMB2 appear to be of similar size to those described by Stibitz *et al.* (1988a). However, a small 0.3kb *EcoRI* fragment, which together with the two larger *EcoRI* fragments made up the *vir* locus described by Stibitz *et al.* (1988a), was not present in pRMB2 and did not appear to be necessary for complementation of BP347. Stibitz *et al.* (1988a) reported that the *fha* locus mapped close to the *vir* locus. Comparison of the restriction map of pRMB2 with that of clone pUW21-26 shows an homologous 15.2kb region encoding *vir* and the *fhaB* gene (E3, E2 and E6), but the remaining 10.9kb of pRMB2 (E5, E4 and E1) has a different restriction pattern. The map of pUW21-26 shows restriction sites for *ClaI*. However no restriction sites for this enzyme were found in clone pRMB2, confirming that the two clones contain a non-homologous region.

Hybridisation studies using the E3 fragment of pRMB2 as a probe showed a 22kb *ClaI* fragment for *B.pertussis* strains L84 I, Tohama I and TAB I, and a slightly larger fragment (due to Tn5 insertion) for BP347. The possibility that the differences in the restriction maps of clones pRMB2 and pUW21-26 had arisen as a result of differences in the original strains used to construct the genomic libraries (TAB I and Tohama I respectively) was therefore eliminated. The 2.7kb *EcoRI* fragment was contained within *BamHI* and *ClaI* fragments of approximately 14kb and 22kb respectively in strain TAB I, which was in agreement with the map of the *vir* region presented by Stibitz *et al.* (1988a), and implied that the genomic insert of clone pRMB2 comprised a non-contiguous region of TAB I DNA. This

Figure 5.1.

Restriction maps of the *vir* regions of clones pRMB2 and pUW21-26.

The restriction map of clone pRMB2 is compared with a recently published map of a clone, pUW21-26, which encompasses the *B.pertussis vir* and *fha* loci (Stibitz *et al.*, 1988a).

The hatched area of pRMB2 represents the 8.0kb region which is also contained in pDM1, and encodes the *vir* locus. Asterisks denote the 2.7kb *EcoRI* fragment which contains the site of Tn5 insertion in BP347. The shaded bar represents the region of homology between the two maps.

Stibitz *et al.* (1988a) used transposon mutagenesis of clone pUW21-26 to define the *vir* locus, the structural gene for FHA (*fhaB*) and genes that are possibly required for the synthesis and export of FHA (*fhaA* and *fhaC*). The direction of transcription has been determined for *fha* (Stibitz *et al.*, 1988a) and *vir* (Arico *et al.*, 1989), as indicated by arrows.

E, *EcoRI*; B, *BamHI*; C, *ClaI*.

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[illegible]
$$\frac{vir}{\overline{\phantom{vir}}} \quad \frac{\overline{\phantom{phaA}}}{\overline{\phantom{phaB}}}$$




was confirmed by hybridisation analysis which showed that the Bg5 fragment of pRMB2 (which spans the junction between the regions showing homology and non-homology to pUW21-26) hybridised to two fragments of genomic DNA in *B.pertussis* strains L84 I, BP347, Tohama I and TAB I.

Complementation studies with Tn5 mutant BP353 (Fha-) provided further evidence that pRMB2 shared homology with pUW21-26 only for the region up to and including the *fhaB* gene. The Tn5 insertion in BP353 was contained within the *fhaA* gene (Stibitz *et al.*, 1988a). Clone pRMB2 was unable to restore FHA activity to strain BP353, confirming that it did not encode the sequence necessary to complement the Fha- mutation (i.e. the *fhaA* gene).

#### 5.1.6. Nucleotide sequence analysis of *vir*: homology with bacterial sensory transduction systems.

The nucleotide sequence of the *vir* locus, which was renamed *bvg* for *Bordetella virulence* gene, has recently been determined (Arico *et al.*, 1989). Three ORFs were identified: *bvgA*, *bvgB* and *bvgC*, predicted to encode proteins of 23kD, 30kD and 102kD respectively. A series of non-polar insertion mutations generated in the *bvg* operon indicated that all three genes (*bvgABC*) were required for expression of virulence genes in *B.pertussis* (Arico *et al.*, 1989).

Analysis of the deduced amino acid sequence identified a putative N-terminal signal peptide in BvgB, and a putative transmembrane region in BvgC. This suggested that BvgB was located in the periplasmic space and BvgC traversed the cytoplasmic membrane (Arico *et al.*, 1989). The predicted gene products of *bvgA* and *bvgC* shared extensive homology with several two-component regulatory systems present in pathogenic and non-pathogenic bacteria (Arico *et al.*, 1989). As described in Section 2.6, these systems

consist of a sensor protein which responds to environmental stimuli and transduces signals to its cognate regulator protein, which in turn effects the response. These effector functions usually involve transcriptional regulation or some other cellular function. The family of sensor proteins share an homologous C-terminal transmitter domain, and the family of regulator proteins share an homologous N-terminal receiver domain (reviewed by Stock *et al.*, 1989).

The *B.pertussis* system consisted of three components, one of which (BvgB) showed no homology to sensor or regulator proteins (Arico *et al.*, 1989). A similar three component system has been described for the uptake of hexose phosphates in *E.coli* (Weston and Kadner, 1988). The predicted BvgC polypeptide was unusual: the central portion shared homology with a family of transmitter domains (with the region extending towards the N-terminus sharing extensive homology with the FixL and VirA sensor proteins) however, the C-terminus of BvgC shared homology with receiver domains (Arico *et al.*, 1989). The N-terminus of the predicted BvgA protein shared homology with receiver domains. The predicted C-terminal sequence of BvgA indicated that it belonged to the same subclass of response regulators as FixJ and UhpA, suggesting a similar role for BvgA as a transcriptional activator.

Arico *et al.* (1989) proposed a model for the mechanism of interaction of the *bvg* gene products (see Figure 5.2). Under normal conditions, the transmitter domain of BvgC activates BvgA by phosphorylation of the N-terminal receiver domain. The phosphorylated BvgA protein effects its response by activating transcription of virulence genes. BvgB may exert its effect by interacting directly with the N-terminus of BvgC, or by inactivating an inhibitor of BvgC. The effect of modulators could be

Figure 5.2.

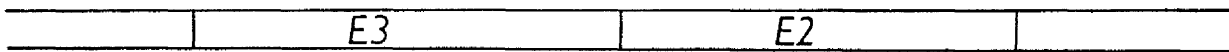
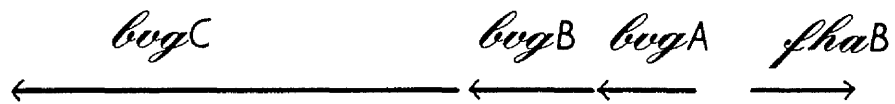
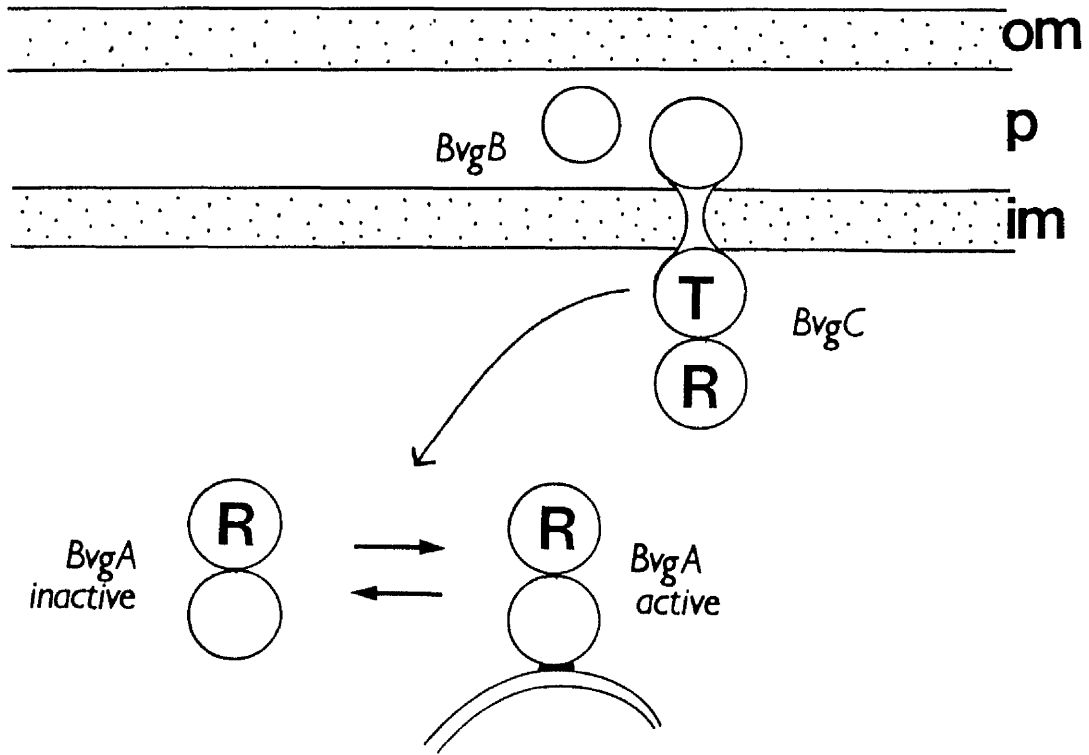
**Model for the mechanism of interaction of the *bvg* gene products.**

This model is adapted from Arico *et al.* (1989). Nucleotide sequence analysis of the *vir* (*bvg*) region encompassing the 2.5kb and 2.7kb *EcoRI* fragments (E2 and E3 respectively), identified three tandemly arranged genes, *bvgA*, *bvgB* and *bvgC*. The predicted *bvgA* and *bvgC* gene products share extensive homology with a family of two-component regulatory proteins.

In this model, the transmitter domain of BvgC activates (by phosphorylation) the N-terminal receiver domain of BvgA, which effects its response as a transcriptional activator of *B.pertussis* virulence genes. The inhibitory effect of modulators, such as MgSO<sub>4</sub> and nicotinic acid, could be mediated by an interaction with BvgB and / or the N-terminus of BvgC in the periplasm. The C-terminal receiver domain of BvgC may act by regulating the activity of the transmitter domain, or may play a similar role to BvgA in DNA binding and transcriptional activation.

T, transmitter domain; R, receiver domain.

om, outer membrane; p, periplasm; im, inner membrane.



mediated by an interaction with BvgB and / or BvgC. The role of an additional receiver domain at the C-terminus of BvgC is presently unknown. Arico *et al.* (1989) propose that it may be involved in regulating the activity of the transmitter domain of BvgC, or may play a role in DNA binding and transcriptional activation, similar to BvgA.

Other studies have shown that regulation of expression of *B.pertussis* genes occurs at the level of transcription: Nicosia and Rappuoli (1987), Knapp and Mekalanos (1988), Melton and Weiss (1989), Miller *et al.* (1989) and Roy *et al.* (1989, 1990).

Nicosia and Rappuoli (1987) found that Vir- BP347 did not produce detectable levels of PT mRNA, which indicated that a functional *vir* (*bvg*) gene was necessary for transcriptional activation of the *ptx* operon. Roy *et al.* (1989) constructed a *B.pertussis* strain which contained a chromosomal in-frame deletion mutation in *bvgA*. This strain (BP3703) was Vir-, which indicated that *bvgA* was required for the expression of virulence determinants in *B.pertussis*. A virulent phenotype was restored to BP3703 when the wild-type *bvgA* gene was provided in-*trans* in clone pCR436. However this clone was unable to confer a Vir+ phenotype to Vir- mutant BP359 which contained a Tn5 insertion in *bvgA*. This suggested that the Tn5 insertion in BP359 had a polar effect on expression of *bvgB* and *bvgC*, and confirmed findings by Arico *et al.* (1989) that all three *bvg* genes were necessary for expression of virulence-associated factors in *B.pertussis* (Roy *et al.*, 1989). Strains of BP3703 ( $\Delta$ *bvgA*) which contained CAT fusions to the promoter region of *fhaB* and *ptx* were constructed. Transcription of both *fhaB* and *ptx* in BP3703 was significantly reduced which confirmed that BvgA was necessary for transcriptional activation of virulence-associated genes in *B.pertussis* (Roy *et al.*, 1989). These authors also demonstrated that

transcriptional activation of the *B.pertussis fhaB* gene in *E.coli* could be achieved by overexpression of *bvgA* alone, which confirmed that *bvgA* encoded a transcriptional activator.

Roy *et al.* (1990) examined the transcriptional regulation of the *bvgABC* operon, and found that it was autogenously activated. Primer extension analysis was used to map transcriptional initiation sites upstream of *bvgA*. Two promoters were identified: a positively autoregulated *bvg* promoter located 90bp upstream of *bvgA* and a *bvg* promoter located 141bp upstream of *bvgA*. These authors proposed a model for the transcriptional regulation of the *bvg* operon: under normal conditions the *bvg* promoter is activated by BvgA in conjunction with RNA polymerase, and transcription initiation from the *bvg* promoter is repressed. Modulators may exert their effect by producing an inactive form of BvgA that no longer functions as a transcriptional activator, which results in a loss of *bvg* promoter activity. Under modulating conditions the *bvg* promoter would be derepressed, which would result in a low level of expression of the *bvg* operon (Roy *et al.*, 1990).

The *mod* mutations described by Knapp and Mekalanos (1988) which resulted in constitutive expression of virulence determinants in *B.pertussis* may have been located in the *bvgA* gene.

## Section 5.2. REGIONS HOMOLOGOUS TO VIR IN THE OTHER BORDETELLA SPECIES.

Hybridisation analysis showed that the *vir* locus was strongly conserved in *B.pertussis*, *B.parapertussis* and *B.bronchiseptica*, however although *B.avium* chromosomal DNA showed homology to the *vir* locus, a markedly different restriction pattern was observed for the homologous fragments.

### 5.2.1. The *vir* region of the mammalian *Bordetella* species.

When pRMB2, and the more specific E3 fragment, were used as probes against *EcoRI*-digested *Bordetella* DNA, *B.pertussis*, *B.parapertussis* and *B.bronchiseptica* showed similar patterns of hybridisation. This provided evidence that the latter two species contained a structural gene for *vir*.

No detectable physical difference, such as inversion or deletion, was found with *EcoRI*-digested DNA from virulent and avirulent phase variant strains of both *B.pertussis* (strains 44122/7R and L84) and *B.bronchiseptica* (strain FW5) when probed with pRMB2 or pDM30. This is consistent with another report which showed that phase variation in one series of avirulent variants of *B.pertussis* strain Tohama arose by spontaneous frame-shift mutations within the 2.7kb *EcoRI* region of the *vir* locus (Stibitz *et al.*, 1989). However with phase variant 11615, the 2.5kb *EcoRI* fragment, which is also part of the *vir* locus, was replaced by a higher molecular weight band. The avirulent phenotype of this strain may have arisen as a result of an insertion into, or rearrangement of, this region. This might suggest that not all phase variants necessarily arise by spontaneous frame-shift mutations.

When pRMB2 was used as a probe against *EcoRI*-digested genomic DNA, additional high molecular weight bands were detected, which differed in

size for each of the three species. Similarly, with *Bam*HI-digested genomic DNA, additional bands of 4.2kb, 6.4kb and 7.5kb were detected in samples of *B.pertussis*, *B.parapertussis* and *B.bronchiseptica* respectively. The most likely explanation is that these additional bands, which were not detected with the *vir*-specific E3 probe, had arisen as a result of hybridisation of pRMB2 to a region elsewhere in the *Bordetella* chromosome. The possibility that these additional bands represented homologous plasmid DNA sequences was ruled out: they were not detected on hybridisation with a pLAFR1 probe.

The E3 probe hybridised to a similar 2.7kb fragment in samples of *Eco*RI-digested *B.pertussis* and *B.parapertussis* DNA. However, the homologous band exhibited in samples of *B.bronchiseptica* DNA was marginally larger. This was more obvious when *Bam*HI-digested genomic DNA was probed with E3: the band detected in samples of *B.pertussis* was 14kb, whereas samples of *B.parapertussis* and *B.bronchiseptica* exhibited bands of 16kb. This implied that the structure of the *vir* region in the latter two species differed from that of *B.pertussis*. A recent report by Monack *et al.* (1989b) has also indicated that the *vir* loci of *B.pertussis* and *B.bronchiseptica* are structurally different. These authors, in agreement with this study, showed that the 2.5kb and 2.7kb *Eco*RI fragments of the two loci appeared to be similar. However, a number of restriction fragment length polymorphisms were found between the flanking regions of the *B.pertussis* and *B.bronchiseptica* *vir* loci (Monack *et al.*, 1989b). A restriction map of the *vir* region in *B.bronchiseptica* showed that the *vir* locus was contained in a 23.5kb *Bam*HI fragment (Monack *et al.*, 1989). From hybridisation data, this *Bam*HI fragment for *B.bronchiseptica* strains FW5, AS1 and 276 was estimated to be only 16kb. However since this size (16kb) was calculated from a graph of migration distance plotted against molecular size of lambda DNA markers,



it may have been an underestimation of the actual size.

When *Bam*HI-digested genomic DNA was probed with pRMB2, the end regions of the genomic insert of pRMB2 hybridised to genomic fragments of 12kb and 14kb for samples of *B.pertussis*, but fragments of 6.5kb and 16kb for samples of *B.parapertussis* and *B.bronchiseptica*. This finding agrees with other work which has shown that *B.parapertussis* and *B.bronchiseptica* share a very close genetic relationship with *B.pertussis* showing only limited divergence (Musser *et al.*, 1986; Arico and Rappuoli, 1987; Gross *et al.*, 1989).

Recent studies have shown that the *vir* loci of *B.parapertussis* and *B.bronchiseptica* were able to *trans*-activate the *ptx* operon, which indicated that the *vir* locus contained in both species was functional (Gross and Rappuoli, 1988; Lee *et al.*, 1989; Monack *et al.*, 1989a).

#### 5.2.2. Homology to *vir* in *B.avium* strains.

*Eco*RI-digested DNA from *B.avium* strains showed a markedly different hybridisation pattern to the other *Bordetella* species. Hybridisation of DNA from virulent *B.avium* strains with pRMB2 produced bands of 17kb, 5.3kb and a faint band of 4.9kb; E3 hybridised only to the 4.9kb fragment. The lower intensity of the 4.9kb band in the virulent *B.avium* samples, compared to homologous bands in the *B.pertussis* samples, suggested that the 4.9kb *Eco*RI fragment in virulent *B.avium* strains shared only partial sequence homology with E3. Additional bands present in some of the samples when pRMB2 was used as a probe also hybridised to a pLAFR1 probe, which indicated that these strains probably contained plasmids with sequences homologous to pLAFR1.

Avirulent *B.avium* strains (GOBL110 and GOBL122) produced a slightly

different hybridisation pattern. The pRMB2 probe hybridised to genomic *EcoRI* fragments of 17kb and 5.2kb; E3 hybridised to the 5.2kb fragment. As it was not known whether these avirulent *B.avium* strains were derived from virulent strains, it was not possible to correlate the physical changes with phenotypic changes. Strains GOBL110 and GOBL122 were described as "*B.avium*-like", and may have been assigned to the species on the basis of phenotypic characteristics. The differences in hybridisation patterns may therefore be associated with a divergence of strains GOBL110 and GOBL122 from typical *B.avium* strains. Additional information is required before any conclusions can be drawn from the differences observed between the virulent and avirulent strains analysed.

Only one *B.avium* strain (4091) was further analysed with *BamHI*: with pRMB2 as a probe a single band was detected, which confirmed the distinctiveness of *B.avium* from the other *Bordetella* species. No hybridisation to the E3 probe was detected in the *BamHI*-digested *B.avium* sample, however since the E3 probe only produced a faint band in *EcoRI*-digested samples, perhaps a longer exposure to autoradiography would have been necessary to detect hybridisation.

The distinctiveness of *B.avium* from the other *Bordetella* species was not entirely unexpected. Other studies have also indicated that *B.avium* does not share a close genetic relationship with the other *Bordetella* species (Kerstens *et al.*, 1984; Musser *et al.*, 1986; see also Section 2.1.4). Gentry-Weeks *et al.* (1988) provided preliminary evidence that in response to  $MgSO_4$ , *B.avium* undergoes the phenotypic modulation characteristic of the other *bordetellae*, indicating that *B.avium* may have a functionally similar *vir* locus. *B.avium* produces the fewest virulence-associated factors and, as suggested by Wardlaw and Parton (1988a), may not

therefore suffer any major disadvantage by their constitutive expression under all growth conditions. Further studies are necessary to establish if *B.avium* has a functional *vir* locus. It would be of interest to clone and characterize the sequences of *B.avium* DNA showing homology to the *B.pertussis* *vir* probes, to determine whether they could encode a similar functional *vir* locus.

### Section 5.3. COMPLEMENTATION OF AVIRULENT PHASE VARIANT STRAINS.

#### 5.3.1. Complementation of an avirulent phase variant *B.pertussis* strain.

In-*trans*, the *vir* locus encoded by pDM1 was able to restore expression of virulence factors to *B.pertussis* L84 IV. This was consistent with a report by Brownlie *et al.* (1988) which indicated that pRMB2 was able to *trans*-complement the strain. These findings confirmed that loss of expression of virulence determinants in the phase IV strain had arisen as a result of mutational events in the *vir* locus. Hybridisation studies indicated that there was no detectable physical change in the *vir* region of strain L84 IV, which suggested that the avirulent phenotype of this strain may have arisen by a spontaneous frame-shift mutation similar to that described by Stibitz *et al.* (1989). The mutation described by these authors was contained in a 1.4kb *SalI* fragment, and the E3 fragment in clones pDM1 and pRMB2 encompassed this region.

### 5.3.2. Complementation of avirulent phase variants of *B.bronchiseptica*.

Hybridisation studies demonstrated that *B.bronchiseptica* contained a *vir* region homologous to that in *B.pertussis*. The expression of virulence determinants in *B.bronchiseptica* is affected by phase variation (Lax, 1985) and it was of interest to determine whether avirulent phase variant strains of *B.bronchiseptica* also arose as a result of genotypic changes in *vir*.

Clone pDM1 restored a Vir+ colonial morphology (i.e. small, domed) to *B.bronchiseptica* strains AS1 III, FW5 III and 276 III, whereas transconjugants containing the control plasmid (pLAFR1) retained a Vir- colonial morphology (i.e. large, flat). This demonstrated that phase variation in *B.bronchiseptica* also occurs as a result of mutations in *vir*.

Since a comparison of phase I with phase III *B.bronchiseptica* FW5 DNA revealed no detectable physical alterations in the *vir* region (Section 4.3.1), the avirulent phenotype of this strain *may have arisen* as a result of a frame-shift mutation similar to that described by Stibitz *et al.* (1989) for *B.pertussis*. The *vir* regions of *B.bronchiseptica* strains AS1 III and 276 III were not investigated. In a recent study, Monack *et al.* (1989b) examined the *vir* regions of fifteen avirulent phase variant *B.bronchiseptica* strains: nine contained no obvious physical change and may have arisen as a result of a single frame-shift mutation, however the remaining six strains contained deletions (ranging in size from 50bp to 500bp). These findings supported hybridisation data which indicated that the avirulent phase variant *B.pertussis* strain 11615 had arisen as a result of a rearrangement in *vir*. Therefore it appears that the genomic changes in *vir* that give rise to avirulent phase variants, can occur by a variety of mechanisms in both *B.pertussis* and *B.bronchiseptica*.

The *B.pertussis* *vir* locus encoded by pDM1 restored a Vir+ colonial

morphology to the three Vir- phase variant *B.bronchiseptica* strains, which demonstrated that the *vir* loci from the two species function in a similar manner. However, haemolysin activity was detected in <sup>variable</sup> a percentage of the phase III *B.bronchiseptica* pDM1 transconjugants. This suggested that perhaps the *B.pertussis vir* gene product was only weakly active in *B.bronchiseptica*, such that only a low level of expression of virulence determinants was restored. This hypothesis was substantiated by the finding that both the Hly+ and Hly- pDM1 transconjugant colonies were positive in a qualitative AC assay, which indicated that expression of AC had been restored to all the pDM1 transconjugants. It would have been of interest to determine the AC activity quantitatively, and compare the levels between phase I strains, phase III strains and phase III (pDM1) transconjugant strains. FHA activity was also restored to the three *B.bronchiseptica* phase III strains by pDM1. However, the haemagglutination titres for the *B.bronchiseptica* phase III (pDM1) strains were much lower than those for *B.pertussis* phase I strains. Since the corresponding *B.bronchiseptica* phase I strains were not included in these assays, no definite conclusions could be drawn. However, these preliminary experiments suggested that expression of FHA in *B.bronchiseptica* phase III strains was also restored to only a low level by pDM1.

The above findings agreed with work by Monack et al. (1989b) which showed that the *B.pertussis vir* locus could *trans*-complement Vir- phase variant *B.bronchiseptica* strains, but produced lower levels of HLY expression. However, these authors also observed a similar effect when the *B.bronchiseptica vir* locus was provided *in-trans* in both Vir+ and Vir- *B.bronchiseptica* strains. Monack et al. (1989b) suggested that the lower levels of *vir*-mediated activity in these strains resulted from a copy

number effect, and proposed that multiple copies of *vir* in *B.bronchiseptica* resulted in increased levels of *vir*-encoded polypeptides which caused decreased levels of transcriptional activation of the *vir* operon i.e. sensitive autoregulation. Thus the mechanisms of regulation of expression of virulence determinants in the two species may be different.

Since the *B.bronchiseptica vir* locus is physically different from that of *B.pertussis*, and appears to be regulated in a different manner, it would be of interest to clone and perform extensive molecular and genetic analysis of the *B.bronchiseptica vir* locus, to elucidate the mechanisms of its transcriptional activation. Examination of the *vir* region of avirulent phase variant *B.bronchiseptica* strains should provide further information on the molecular mechanisms of phase variation in *B.bronchiseptica*.

#### Section 5.4. THE EFFECT OF MULTIPLE COPIES OF VIR ON THE RESPONSE TO MODULATORS.

In response to modulators, expression of multiple virulence determinants in *B.pertussis* is concomitantly lost in an effect mediated by *vir*. Vector pLAFR1 has a copy-number of 4 to 5 in *E.coli* (Friedman *et al.*, 1982), and it was of interest to determine whether the presence of multiple copies *in-trans* of the cloned *vir* locus in *B.pertussis* would affect the response to modulators.

Expression of HLY was lost from *B.pertussis* strains L84 I, Tohama I, BP347 (pRMB2) and BP347 (pDM1) when grown at low temperature. Therefore the multiple copies of *vir* present in the latter two strains had no detectable effect on the overall response. Melton and Weiss (1989) examined the levels

of transcription of *vir*-regulated genes in *B.pertussis* at various temperatures, and found a direct relationship between temperature and levels of mRNA. However, the levels of transcription were also reduced at low temperatures for non-*vir*-regulated genes, which suggested that the low level of expression of virulence genes at low temperatures was coincidental, and not directly mediated by *vir*.

In response to nicotinic acid, HLY activity was lost from *B.pertussis* strain L84 I, but not from strains Tohama I, BP347 (pRMB2) and BP347 (pDM1). The observation that some *B.pertussis* strains appear to be less responsive than others to modulation by nicotinic acid has also been reported by McPheat *et al.* (1983), Armstrong and Parker (1986) and Melton and Weiss (1989).

Although BP347 was derived from strain Tohama, the *vir* locus encoded by clones pRMB2 and pDM1 was from strain TAB, which responds to modulation by nicotinic acid (Brownlie *et al.*, 1985a). These findings suggested that the lack of response of some strains to nicotinic acid modulation was probably not associated with the *vir* gene product. Perhaps some strains have structurally different outer membranes which renders them resistant to penetration by nicotinic acid.

Melton and Weiss (1989) demonstrated that modulation by MgSO<sub>4</sub> eliminated transcription of *vir*-regulated genes, and reduced transcription of *vir* itself, which suggested that global regulation of expression of virulence genes was obtained by modifying expression of *vir*. These findings were consistent with the possible mechanisms of *vir* regulation presented by Arico *et al.* (1989) and Roy *et al.* (1990), as described in Section 5.1.6. In view of these findings, it would be of interest to determine in a qualitative assay whether multiple copies of *vir* in-trans in clones pDM1

and pRMB2 had any effect on MgSO<sub>4</sub>-induced modulation in *B.pertussis*.

4

## Section 5.5. EXPRESSION IN *E.COLI* OF *VIR*-REGULATED *B.PERTUSSIS* GENES.

### 5.5.1. Expression in *E.coli* of FHA.

At this stage in the study the restriction map of pRMB2 had not been determined, therefore a haemagglutination assay was performed on *E.coli* DH1 (pRMB2) to provide preliminary information as to whether the genomic insert of pRMB2 encompassed both the *vir* and *fha* loci. No haemagglutinating activity was detected, which implied that FHA was not present on the bacterial cell surface.

Stibitz *et al.* (1988a) assayed production of *B.pertussis* FHA in *E.coli* by colony blots with anti-FHA antiserum. Cosmid clones containing *vir* and the entire *fha* region (e.g. pUW21-26) directed the production of a substance which was immunologically cross-reactive to FHA. However, cosmids containing the *fha* region alone did not produce iFHA. These studies demonstrated that *vir* could be expressed in *E.coli* without the use of a heterologous promoter, and confirmed the dependence on *vir* for expression of FHA in *E.coli*. Further analysis showed that inactivation by Tn-mutagenesis of *fhaA* in pUW21-26 caused an apparent overproduction of FHA in *E.coli* (Stibitz *et al.*, 1988a). A role was proposed for *fhaA* in being involved in the association of FHA with the bacterial cell surface, and the assay used by these authors to detect FHA in *E.coli* measured production of extracellular FHA, which would not have been affected by inactivation of *fhaA*. Inactivation by Tn-mutagenesis of the *fhaC* region of clone pUW21-26 eliminated the detection of iFHA in *E.coli*, which implied that *fhaC* was



required for the proper export of FHA to the outside of the cell (Stibitz *et al.*, 1988a).

Comparison of the restriction map of pRMB2 with that of clone pUW21-26 (see Section 5.1.5.) showed that pRMB2 contained the *vir* (fragments E2 and E3) and *fhaB* (fragment E6) genes, but not the *fhaA* and *fhaC* genes. Therefore although pRMB2 contained the necessary sequence to induce expression of *fhaB*, the FHA protein would not have been exported to the outside of the cell and assembled by *E.coli* due to the lack of the *fhaC* and *fhaA* genes respectively. Perhaps immunologically-reactive FHA polypeptides would have been detected in a cell lysate of *E.coli* DH1 (pRMB2). It would be of interest to perform Northern blot analysis of *E.coli* DH1 (pRMB2) with *vir*-specific and *fhaB* specific probes to determine whether the two loci are transcribed by *E.coli*.

#### 5.5.2. Expression in *E.coli* of AGGs 2 and 3.

Since Vir- BP347 was derived from strain Tohama (ST 1,2), a preliminary analysis was performed to determine whether pRMB2 could restore expression of these AGGs to BP347. Expression of the non-fimbrial AGG 1, but not the fimbrial AGG 2, was restored to BP347 by pRMB2. However, further analysis indicated that the Tn5 mutant BP353, which was generated at the same time as BP347, was negative for AGG 2 expression. This implied that the Tohama-derived strain, BP338, which was used to create these Tn mutants (Weiss *et al.*, 1983) had undergone serotype variation, such that even in the presence of *vir* production of serotype 2 fimbriae was not detected. The *fim2* gene encoded by pIL22 was derived from strain Wellcome 28 (ST 1,2,3) (Livey *et al.*, 1987) and was therefore presumed to contain a functional *fim2* gene.

The ability of the *vir* locus encoded by pRMB2 to *trans*-activate the

*fim2* gene cloned in a compatible plasmid in *E.coli*, was assayed by slide agglutination with "Preston 2" antiserum. No agglutinating activity was detected. This was not entirely unexpected, since a positive reaction would have required that the ST2 subunit was secreted by *E.coli*, and assembled on the cell surface in the proper conformation, and in sufficient density for bivalent antibody to cross-link two bacteria.

Using lysed whole-cell samples, production of the ST2 fimbrial subunit in *E.coli* JA221 (pIL22, pRMB2) was assayed by ELISA. An immunoreactive product was detected with polyclonal "Preston 2" antiserum, but not with monoclonal Ag2A antiserum. However, the fact that the *E.coli* negative control strains JA221 (pRMB2) and JA221 (pDM1) also reacted positively with the "Preston 2" antiserum implied that an antigen other than the ST2 fimbrial subunit was being detected in all three *E.coli* samples.

When the SDS-PAGE protein profile of *E.coli* JA221 (pIL22, pRMB2) was compared with profiles of the control *E.coli* strains containing pIL22 alone or pRMB2 alone, no additional polypeptide which may have been due to production of the ST2 fimbrial subunit was detected. A prominent polypeptide which was present in samples of *E.coli* strains JA221 (pIL22, pRMB2) and JA221 (pIL22) was presumed to be encoded by vector pBR328.

Immunoblot analysis with "Preston 2" antiserum failed to detect production of the ST2 fimbrial subunit protein, or precursor protein, in the *E.coli* strain which contained both the *vir* and *fim2* clones. In all three *E.coli* samples an immunoreactive polypeptide of 35kD was detected, which confirmed the ELISA cross-reactivity of "Preston 2" antiserum with proteins native to *E.coli*.

Assuming that the *vir* locus encoded by pRMB2 was functional in *E.coli*, there are several reasons which may explain why immunoreactive ST2 fimbrial

subunits were not detected in *E.coli* JA221 (pIL22, pRMB2). For example, the mRNA or polypeptides produced may have been unstable, or the polypeptides may have been produced with a different conformation to the wild-type ST2 fimbrial subunits.

Walker *et al.* (1990) obtained a high level of expression in *E.coli* of the *B.pertussis* ST2 fimbrial subunit from a subclone of pIL22. Expression of the *fim2* gene was under the control of lambda promoters and an *E.coli* translational initiation region. Expression of *fim2* was best detected in lon protease and heat shock protein deficient *E.coli* strains. Although the ST2 fimbrial subunit was transported across the inner membrane, no fimbriae were detected on the surface of the *E.coli* cells, which suggested that the assembly in *E.coli* of native ST2 fimbriae may require additional components (Walker *et al.*, 1990).

Northern blot analysis of *E.coli* containing pIL22 and pRMB2, with a *fim2* specific probe would provide valuable information as to whether the *fim2* gene is *trans*-activated by *vir*.

Three pIC20H derived subclones of pDM1 (pDM623, pDM30 and pDM62) were introduced into *E.coli* DH1 containing the *fim3* gene (encoded by p26). Production of the ST3 fimbrial subunit in these three *E.coli* strains was assayed by immunoblotting with "Preston 3" antiserum. No immunologically reactive polypeptides were detected. Clone pDM623 contained the entire *bvgABC* region (fragments E2 and E3) plus the 2.8kb *BamHI* to *EcoRI* fragment upstream of E2. Miller *et al.* (1989) demonstrated that the entire *bvg* operon was required for *trans*-activation in *E.coli* of the *fhaB* gene, therefore clone pDM623 would have been the most likely to have induced expression of the *fim3* gene. Clone pDM30 contained only the 3' portion of the *bvgC* gene (fragment E3), and would not have been able to *trans*-activate

transcription of *fim3*. Clone pDM62 contained *bvgA*, *bvgB* and the 5' region of *bvgC* (fragment E2) plus the 2.8kb *BamHI* to *EcoRI* fragment upstream of E2.

The genomic inserts of clones pDM62 and pDM623 were cloned in pIC20H in the same orientation as the *lac* promoter. In these clones, it is possible that the *bvgA* gene was overexpressed, although the 2.8kb region between the vector promoter and the start of the *bvgA* gene may have prevented this. Roy *et al.* (1989) demonstrated that overexpression of *bvgA* alone was sufficient to *trans*-activate *fhaB* in *E.coli*. If BvgA was overproduced in *E.coli* strains containing pDM62 or pDM623, it was apparently insufficient to *trans*-activate *fim3*, as determined by immunoblot analysis. Northern blot analysis would provide further information as to whether *fim3* is *trans*-activated by *vir* in *E.coli*.

It would be of interest to clone the E2 fragment (which contains *bvgA*) and the E2 plus E3 fragments (*bvgABC*) into vector pIC20H, to determine *vir* activity in *E.coli* under the transcriptional control of the *lac* promoter.

#### 5.5.3. Expression in *E.coli* of *B.pertussis* AC.

Introduction of *vir* *in-trans* into *E.coli* CAA8306 harbouring the cloned *B.pertussis* *cya* gene had no marked effect on expression of AC enzymic activity. Both pLAFR1-derived and pIC20H-derived *vir* clones failed to activate production of AC.

In agreement with work by Brownlie *et al.* (1988) and Rogel *et al.* (1989), calmodulin-responsive AC enzymic activity was produced from clones pRMB3 and pRMB9, which both contained the *B.pertussis* *cya* gene under the transcriptional control of the *lac* promoter of vector pIC20H. These findings indicated that it was possible to express AC enzymic activity in

*E.coli*. Clone pRMB12 also contained the *cya* gene cloned in pIC20H in the same orientation as the *lac* promoter, but did not produce significant levels of AC enzymic activity in *E.coli* CAA8306. This may have been because the genomic insert of pRMB12 contained sequence 5' to the start of the protein coding region (see Figure 4.34).

In conclusion, the cloned *vir* locus *in-trans* in *E.coli* was unable to induce expression of the *B.pertussis* *fhaB*, *fim2*, *fim3* and *cya* genes. It is now known that *vir* activates expression of virulence genes at the level of transcription, therefore Northern blot analysis with virulence-gene specific probes would confirm whether any of the aforementioned genes are *trans*-activated by *vir* in *E.coli*.

Miller *et al.* (1989) and Roy *et al.* (1990) were unable to detect *vir*-dependent expression of *ptx* in *E.coli*. The failure to detect in *E.coli* *vir*-dependent *trans*-activation of *B.pertussis* virulence genes other than *fha*, suggests that the regulatory mechanism for *fha* differs from that of the other *B.pertussis* *vir*-regulated genes. In addition to *vir*, perhaps other factors are required for transcriptional activation in *E.coli* of virulence genes such as *ptx*, *cya* and *fim*. Genetic analysis of the promoter regions of these genes is necessary to determine the *cis*-acting domains required for transcriptional activation.

Roy *et al.* (1990) suggested that due to the close proximity of the *fhaB* and *bvgA* promoter regions, the *bvgABC* operon may have originally evolved to regulate expression of the *fha* gene. Other virulence genes may later have found it advantageous to integrate *trans*-activation by *vir* into their *cis*-acting mechanisms for transcription.

Section 6: REFERENCES.

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